

**THE CLINICAL AND BIOLOGICAL CONSEQUENCES OF
DIFFERENT FLT3 MUTATIONS IN PATIENTS WITH AML**

Adam J. Mead

A thesis submitted for the degree of Doctor of Philosophy

University College London

2009

DECLARATION

I, Adam Mead, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Date: 21st January 2009

ABSTRACT

Characterisation of pathogenic markers in acute myeloid leukaemia (AML) may benefit patients through refinement of risk stratification, application of molecularly targeted therapy and improved understanding of AML biology. Whilst the presence of an internal tandem duplication (ITD) within the *fms*-like tyrosine kinase-3 (FLT3) gene is known to predict adverse outcome in young adults with AML, the clinical significance of activating mutations in the tyrosine kinase domain (TKD) of FLT3 is unclear. Therefore, a highly sensitive and specific denaturing-HPLC technique was developed to screen for FLT3/TKDs in 1339 young adult patients with AML. Mutations were detected in 161 (12%) cases, with a high incidence in patients with *inv*(16) (24%; $P=.009$), a group in which FLT3/ITDs are uncommon. Unlike FLT3/ITDs, FLT3/TKDs were associated with a favourable long-term outcome with a 10-year overall survival (OS) of 36% for FLT3 WT, 51% for FLT3/ITD⁻TKD⁺ and 24% for FLT3/ITD⁺TKD⁻ patients ($P<.001$). The relative FLT3/TKD mutant level was highly variable with the favourable prognosis residing in those patients with greater than 25% mutant alleles (10-year OS of 59%), possibly reflecting the stage at which the mutation is acquired. The mechanism of FLT3 activation also influenced sensitivity to FLT3-inhibitor induced cytotoxicity, with FLT3/ITD⁺ blast cells more sensitive than FLT3/TKD⁺ cells. Following lentiviral transduction, FLT3/ITD-transduced 32Dcl3 and Ba/F3 cells demonstrated more rapid proliferation than FLT3/TKD-transduced cells. In an NB4 cell line model of ATRA-induced myeloid differentiation, the presence of a FLT3/ITD inhibited differentiation unlike a FLT3/TKD mutation which increased differentiation. Furthermore, FLT3/ITD-transduced CD34 positive haematopoietic stem cells showed greater cytokine-free survival of colony forming cells than FLT3/TKD-transduced cells. Signalling studies also revealed that a FLT3/ITD induced stronger STAT5 activation than a FLT3/TKD mutation. This unexpected genotype-phenotype relationship is of direct relevance to current clinical decision making in AML, and may also provide insights into mechanisms of chemoresistance.

ACKNOWLEDGEMENTS

I am grateful to Professors Rosemary Gale, David Linch and Asim Khwaja, under whose mentorship I have begun to understand how to identify the important questions in leukaemia biology, as well as how to address them. These studies would not have been possible without the patients who agreed to enrol onto the MRC clinical trials as well as the clinical investigators who entered and managed these patients. Thanks also to my wife, Marianne, whose extraordinary patience allowed me to spend a number of our “holidays” writing this thesis.

TABLE OF CONTENTS

TITLE PAGE	1
DECLARATION	2
ABSTRACT	3
ACKNOWLEDGEMENTS	4
TABLE OF CONTENTS	5
LIST OF FIGURES	12
LIST OF TABLES	15
COMMONLY USED ABBREVIATIONS	17
 CHAPTER 1: INTRODUCTION	 18
1.1 Clinical presentation of AML	18
1.2 Diagnosis and classification of AML.....	19
1.2.1 French American British (FAB) classification	19
1.2.2 Secondary and therapy related AML	19
1.2.3 Recurrent cytogenetic abnormalities in AML.....	19
1.2.4 World health organisation (WHO) classification.....	20
1.3 Prognostic factors in patients with AML	20
1.3.1 Clinical Prognostic Factors	23
1.3.2 Cytogenetic Risk Groups	23
1.3.3 Combining different prognostic factors	24
1.3.4 Molecular Prognostic Factors	25
1.4 Conventional Chemotherapeutic Approaches in AML.....	26
1.4.1 Induction Chemotherapy for non-APL AML	26
1.4.2 Consolidation Chemotherapy	27
1.4.3 Autologous stem cell transplantation (SCT).....	27
1.4.4 Allogeneic SCT	27
1.4.5 Acute Promyelocytic Leukaemia (APL).....	28
1.5 Risk-adapted therapy.....	28
1.5.1 Monitoring for minimal residual disease (MRD).....	29
1.6 Molecular and cellular pathogenesis of AML.....	29
1.6.1 Oncogenes	30
1.6.2 Mutations in growth factor receptors in AML	31
1.6.3 Cooperating genetic events in leukaemogenesis.....	32

1.6.4	Leukaemic stem cells	33
1.7	Novel Therapeutic Approaches in AML.....	34
1.7.1	Targeted inhibitors	35
1.7.2	Other novel therapies	35
1.8	Aims of this thesis.....	36
CHAPTER 2: MATERIALS AND METHODS		37
2.1	Cell Culture	37
2.1.1	Cell lines	37
2.1.2	Cell culture general reagents.....	37
2.1.3	Tyrosine Kinase Inhibitors.....	38
2.1.4	Other therapeutic agents.....	38
2.1.5	Recombinant growth factors	38
2.1.6	Cell culture plastics.....	38
2.1.7	Ficoll-Hypaque centrifugation	38
2.1.8	Cell cryopreservation and thawing.....	38
2.1.9	Trypan blue exclusion.....	39
2.1.10	Propidium iodide staining	39
2.2	Molecular Biology	39
2.2.1	Reagents	39
2.2.2	Buffers.....	41
2.2.3	DNA Extraction	41
2.2.4	Polymerase chain reaction (PCR)	42
2.2.5	Agarose gel electrophoresis	42
2.2.6	End-labelling of primers	43
2.2.7	Polyacrylamide gel electrophoresis.....	43
2.2.8	Restriction enzyme digestion	43
2.2.9	DNA Sequencing protocol	43
2.2.10	LB broth and plates	44
2.2.11	Cloning of PCR products	44
2.3	SDS-PAGE, Immunoblotting and Flow Cytometry	45
2.3.1	Reagents	45
2.3.2	Buffers.....	45
2.3.3	Acrylamide gel	46
2.3.4	Antibodies	46

2.3.5	Flow cytometry	47
-------	----------------------	----

CHAPTER 3: SCREENING FOR MUTATIONS IN THE TYROSINE KINASE

DOMAIN OF FLT3	48
3.1 Introduction	48
3.1.1 Structure of FLT3	49
3.1.2 FLT3 ligand.....	51
3.1.3 Mutations in the JM domain of FLT3	51
3.1.4 Mutations in the TK domains of FLT3	52
3.1.5 Screening techniques for the detection of FLT3/TKD mutations.....	53
3.2 Patients, Materials and Methods	56
3.2.1 Patients	56
3.2.2 Screening for FLT3/TKD mutations by dHPLC.....	58
3.2.3 Screening for FLT3/TKD mutations by <i>EcoRV</i> restriction digest	58
3.2.4 Identification of TKD Mutations	61
3.3 Results	61
3.3.1 Sensitivity of dHPLC for the detection of D835Y mutations.....	61
3.3.2 Incidence of FLT3/TKD mutations.....	63
3.3.3 Identification of FLT3/TKD mutations.....	63
3.3.4 Comparison of <i>EcoRV</i> Screening and dHPLC screening for FLT3/TKD mutations.....	70
3.4 Discussion	70

CHAPTER 4: CLINICAL CHARACTERISTICS AND PROGNOSTIC IMPACT OF FLT3/TKD MUTATIONS AND COMPARISON WITH FLT3/ITDs IN YOUNG

ADULTS WITH AML.....	75
4.1 Introduction	75
4.1.1 Clinical characteristics of AML patients with a FLT3/ITD.....	75
4.1.2 Clinical outcome of patients with FLT3/ITD ⁺ AML	81
4.1.3 Clinical features and outcome of patients with FLT3/TKD ⁺ AML	82
4.2 Patients, Materials and Methods	86
4.2.1 Patients	86
4.2.2 Therapy	87
4.2.3 End Points	88
4.2.4 Statistical methods	88

4.3	Results	89
4.3.1	Demographics and presenting clinical features of patients with a FLT3/TKD mutation	89
4.3.2	Comparison of presenting clinical characteristics of FLT3/TKD ⁺ and FLT3/ITD ⁺ patients	89
4.3.3	Relationship between the presence of a FLT3/TKD mutation and clinical outcome in patients with non-APL AML	92
4.3.4	Impact of cytogenetic subgroup on the prognostic impact of FLT3/TKD mutations	93
4.3.5	Analysis of both FLT3/TKD and FLT3/ITD status	93
4.3.6	Multivariate analysis	97
4.4	Discussion	97

CHAPTER 5: THE IMPACT OF FLT3/TKD MUTANT LEVEL ON CLINICAL CHARACTERISTICS AND OUTCOME OF YOUNG ADULTS WITH AML

5.1	Introduction	103
5.1.1	Biological basis for different FLT3/ITD mutant levels	103
5.1.2	Clinical impact of FLT3/ITD mutant level	105
5.1.3	Paired presentation and relapse studies of FLT3/ITD mutations	107
5.2	Methods	109
5.2.1	Characteristics of the cohort studied	109
5.2.2	Quantification of FLT3/TKD mutants by semi-quantitative PCR and denaturing polyacrylamide gel electrophoresis	109
5.2.3	Quantification of FLT3/TKD mutants by fragment analysis	110
5.3	Results	111
5.3.1	Distribution of mutant level	111
5.3.2	Clinical characteristics according to FLT3/TKD mutant level	112
5.3.3	Distribution of FLT3/TKD and FLT3/ITD mutations according to mutant level	113
5.3.4	Clinical outcome according to FLT3/TKD mutant level	113
5.3.5	Paired presentation and relapse samples from FLT3/TKD ⁺ patients	115
5.4	Discussion	115

CHAPTER 6: STUDIES OF THE CYTOTOXIC EFFECT OF FLT3 INHIBITORS AND CYTARABINE ON PRIMARY AML BLAST CELLS

6.1	Introduction	120
6.1.1	Can conventional therapeutic strategies be used to overcome the adverse prognosis associated with FLT3/ITDs?	120
6.1.2	Targeted therapies for human malignancies	122
6.1.3	FLT3 inhibitors	122
6.2	Materials and Methods	128
6.2.1	Patients	128
6.2.2	Reagents	128
6.2.3	Detection and Identification of FLT3 Mutations	128
6.2.4	MTS Assays	128
6.2.5	Sequential incubation with lestaurtinib and cytarabine	129
6.2.6	Data Analysis and Statistical Interpretation	129
6.3	Results	129
6.3.1	Comparison of the cytotoxic effect of PKC412 and lestaurtinib on primary AML blast cells	129
6.3.2	Characteristic of the whole cohort of patients studied	130
6.3.3	The cytotoxic effect of cytarabine on primary AML blast cells with different FLT3 mutation status	130
6.3.4	The cytotoxic effect of lestaurtinib on primary AML blast cells with different FLT3 mutation status	131
6.3.5	The cytotoxic effect of a combination of cytarabine and lestaurtinib on primary AML blast cells with different FLT3 mutation status	132
6.3.6	Sequential incubation of primary AML cells with cytarabine and lestaurtinib	133
6.4	Discussion	136

CHAPTER 7: EXOGENOUS EXPRESSION OF FLT3 MUTANTS IN CELL LINE

MODELS AND HUMAN PRIMARY HAEMATOPOIETIC STEM CELLS		142
7.1	Introduction	142
7.1.1	The role of FLT3 in normal haematopoiesis	142
7.1.2	Signalling events downstream of FLT3/WT activation	143
7.1.3	Transduction of FLT3/ITDs in cell line models	144
7.1.4	Correlation of signalling studies in cell line models with primary AML blast cells	145
7.1.5	Transduction of FLT3/TKDs in cell line models	146

7.1.6	The impact of mutant FLT3 on haematopoietic cell differentiation.....	147
7.1.7	Transduction of FLT3 mutations into normal murine haematopoietic cells	148
7.1.8	Transduction of FLT3 mutations into normal human haematopoietic cells <i>in vitro</i>	149
7.1.9	Aims	150
7.2	Materials and methods	150
7.2.1	Cloning of FLT3 into an MSCV lentiviral vector.....	150
7.2.2	Haemopoietic cell lines	153
7.2.3	Electroporation of plasmid constructs into 32Dcl3 cells	153
7.2.4	Lentiviral production.....	154
7.2.5	Biological titre of lentiviral supernatants.....	155
7.2.6	Lentiviral transduction of haemopoietic cell lines	155
7.2.7	Assessment of cell proliferation.....	155
7.2.8	Cytotoxicity assays	156
7.2.9	Detection of proteins by immunoblotting	157
7.2.10	Assessment of intracellular phospho-proteins by flow cytometry	157
7.2.11	NB4 Cell Differentiation Experiments	158
7.2.12	Primary CD34+ Cells.....	158
7.3	Results	159
7.3.1	Confirmation of FLT3 expression using the cloned plasmid constructs.....	159
7.3.2	Expression of FLT3 in haemopoietic cell lines	160
7.3.3	Impact of transduced FLT3 expression on the proliferation of haemopoietic cell lines.....	161
7.3.4	Effect of cytotoxic agents on FLT3 expressing cell lines.....	167
7.3.5	Signalling events in transduced Ba/F3 cells	169
7.3.6	The impact of FLT3 expression on ATRA-induced differentiation of NB4 cells.....	173
7.3.7	Lentiviral transduction of primary CD34+ cells	175
7.3.8	Colony assays of transduced CD34+ cells	176
7.4	Discussion	178
CHAPTER 8: CONCLUSIONS AND FUTURE DIRECTIONS		184
8.1	Future directions	187

8.2	Conclusions	192
	REFERENCES.....	193

TABLE OF FIGURES

CHAPTER 3

Figure 3.1. (A) Diagrammatic representation of the key structural features of the class III receptor tyrosine kinase FLT3 and (B) the key abnormalities of the two main types of mutation, internal tandem duplications and tyrosine kinase domain mutations	50
Figure 3.2. Schematic representation of FLT3/TKD mutation detection by dHPLP.....	57
Figure 3.3. Theoretical melting curves of PCR products generated by WAVEMAKER software	59
Figure 3.4. dHPLC chromatogram traces of PCR products amplified from mixtures of genomic DNA from a heterozygous D835Y mutant positive case and from the FLT3 wild-type NB4 cell line	62
Figure 3.5. dHPLC chromatogram traces of different FLT3 TKD mutations and their confirmation by specific restriction digest and/or direct sequencing.....	65-69
Figure 3.6. <i>EcoRV</i> restriction digest screening for FLT3/TKD mutations and the corresponding dHPLC chromatograms	71

CHAPTER 4

Figure 4.1. Clinical outcome for non-APL AML patients stratified according to FLT3/ITD and FLT3/TKD status.....	94
Figure 4.2. Cumulative incidence of relapse (A) and overall survival (B) stratified according to karyotype and cytogenetic category.	95
Figure 4.3. Overall survival in non-APL AML patients according to cytogenetic risk group.....	96

CHAPTER 5

Figure 5.1: Possible explanations for a low level mutation accounting for 20% of alleles.....	104
Figure 5.2: Diagrammatic representation of early and late mutations in leukaemogenesis	106

Figure 5.3: Possible explanations for high level FLT3 mutants	107
Figure 5.4: Analysis of the level of 9 D835V mutations using a radiolabelled 20F primer and <i>HincII</i> digestion.	110
Figure 5.5: Anaysis of two D835Y mutants by fragment analysis after <i>ClaI</i> digestion.	111
Figure 5.6. Distribution of the relative FLT3/TKD mutant level in 115 cases.....	112
Figure 5.7: Clinical outcome for non-APL AML patients stratified according to FLT3/TKD mutant level.....	114

CHAPTER 6

Figure 6.1: Cytotoxic dose-response to lestaurtinib and PKC412 of blast cells from 7 patients with AML.....	130
Figure 6.2: Cytotoxic responses to cytarabine stratified according to FLT3 mutation status.....	132
Figure 6.3: Cytotoxic responses to lestaurtinib stratified according to FLT3 mutation status.....	133
Figure 6.4: Scatter plot of the cytotoxic effect of lestaurtinib in combination with cytarabine.	134
Figure 6.5: The impact of different sequences of administration of lestaurtinib on blast cells from 6 patients with AML.	135

CHAPTER 7

Figure 7.1: Duplicated segment of the juxtamembrane domain of the FLT3/ITD vector showing the inserted 20 amino acid sequence that resulted from a 57 bp tandem duplication and 3 bp insertion.	151
Figure 7.2: Plasmids used for FLT3 cloning.....	152
Figure 7.3: Assessment of cell number and viability using flow cytometry with propidium iodide staining and calibration beads.....	156
Figure 7.4: Detection of FLT3 protein (135 kDa) by immunoblotting in 293T cells transduced with the different FLT3 constructs.....	160
Figure 7.5: Western blotting studies to show expression of FLT3 protein in transduced cell lines.....	161
Figure 7.6: Proliferation rates of transduced 32Dcl3 cells.....	162

Figure 7.7: Impact of the addition of 20 ng/ml FL on the proliferation of 32Dcl3 transduced cells over 72 hours.	164
Figure 7.8: Proliferation rates of transduced Ba/F3 cells.....	166
Figure 7.9: Proliferation rates of NB4 and cells transduced with the different constructs.	167
Figure 7.10: Cytotoxic effect of lestaurtinib on FLT3 transduced 32Dcl3 and Ba/F3 cells.....	168
Figure 7.11: The impact of lestaurtinib on Ba/F3 transduced cells assessed by propidium iodide staining after 24 hours.	169
Figure 7.12: Immunoblotting of phospho-signalling proteins in transduced Ba/F3 cells.	170
Figure 7.13: Assessment of intracellular phospho-STAT5 by flow cytometry of fixed and permeabilised transduced Ba/F3 cells	171
Figure 7.14: Results of 3 independent experiments on intracellular phospho-signalling proteins in transduced Ba/F3 cells.....	172
Figure 7.15: ATRA-induced CD11b expression in transduced NB4 cells	174
Figure 7.16: ATRA-induced CD11b expression in transduced NB4 cells and the impact of lestaurtinib.....	175
Figure 7.17: Transduction of CD34+ cells with vector or FLT3 constructs	177

LIST OF TABLES

CHAPTER 1

Table 1.1: The French-American-British (FAB) classification of AML	21
Table 1.2: Recurrent, non-random cytogenetic abnormalities in AML, underlying molecular defect and correlation with morphology	21
Table 1.3: The WHO classification of AML	22
Table 1.4: Prognostic risk groups in AML.....	25

CHAPTER 3

Table 3.1: Members of the class III receptor tyrosine kinase family	48
Table 3.2: Published studies reporting TKD mutations of FLT3 in AML.....	54-55
Table 3.3: PCR primers and restriction enzyme digests used for the detection of FLT3/TKD mutations.....	60
Table 3.4: FLT3/TKD mutations detected in total cohort of 1339 patients.....	64

CHAPTER 4

Table 4.1: Studies of the clinical characteristics of adult AML patients with FLT3/ITDs.	76-78
Table 4.2: Studies of the clinical outcome of adult AML Patients with a FLT3/ITD.	79-80
Table 4.3: Clinical characteristics of patients with FLT3/TKD mutations.....	83
Table 4.4: Clinical Outcome of AML Patients with a FLT3/TKD	84-85
Table 4.5 Clinical and demographic characteristics of 1339 AML patients.....	90
Table 4.6: Incidence of FLT3/TKD ⁺ patients in specific cytogenetic risk groups and subgroups.....	91
Table 4.7: Clinical outcome in FLT3/TKD ⁻ and FLT3/TKD ⁺ non-APL AML patients.	93

CHAPTER 5

Table 5.1: Association of FLT3/ITD Level with Clinical Characteristics and Outcome in non-APL AML	108
Table 5.2: Distribution of FLT3/TKD and FLT3/ITD mutations according to mutant level	113
Table 5.3: Clinical outcome in FLT3/TKD ⁻ and FLT3/TKD ⁺ non-APL AML patients.	115

CHAPTER 6

Table 6.1: Inhibitors of the FLT3 tyrosine kinase	124
Table 6.2: Clinical trials of FLT3 inhibitors in patients with AML	125
Table 6.3: Clinical and demographic characteristics of patients stratified by FLT3 mutation status.....	131
Table 6.4: Sequential culture of blast cells in cytarabine and lestaurtinib.....	135

CHAPTER 7

Table 7.1 Proliferation rates of transduced cell lines.....	163
-------------------------------------------------------------	-----

COMMONLY USED ABBREVIATIONS

AML	Acute myeloid leukaemia	ITD	Internal tandem duplication
APL	Acute promyelocytic leukaemia	LSC	Leukaemic stem cell
ATP	Adenosine triphosphate	MAPK	Mitogen activated protein kinase
ATRA	All-trans retinoic acid	MFI	Median fluorescence intensity
BM	Bone marrow	MLL	Mixed lineage leukaemia
bp	Base pairs	MRC	Medical Research Council
CBF	Core binding factor	MRD	Minimal residual disease
CEBPA	CCAAT/enhancer binding protein- α	MT	Mutant
CIR	Cumulative incidence of relapse	NK	Normal karyotype
CML	Chronic myeloid leukaemia	NPM1	Nucleophosmin
CR	Complete remission	OR	Odds ratio
DFS	Disease free survival	OS	Overall survival
dHPLC	Denaturing high performance liquid chromatography	PB	Peripheral blood
DNA	Deoxyribosenucleic acid cDNA = complementary gDNA = genomic	PBS	Phosphate buffered saline
FAB	French American British	PCR	Polymerase chain reaction
FCS	Foetal calf serum	PI	Propidium iodide
FL	FLT3 ligand	R10	RPMI cell culture media with 10% FCS
FLT3	Fms-like tyrosine kinase-3	RD	Resistant disease
GFP	Green fluorescent protein	RR	Relapse rate
GFR	Growth factor receptor	RTK	Receptor tyrosine kinase
HPC	Haematopoietic progenitor cell	SCT	Stem cell transplant
HSC	Haematopoietic stem cell	STAT5	Signal transducer and activator of transcription 5
IL-3	Interleukin-3	TKD	Tyrosine kinase domain
JM	Juxtamembrane	TRM	Treatment related mortality
		WBC	White blood cell count
		WHO	World Health Organisation
		WT	Wild type

CHAPTER 1: INTRODUCTION

Acute myeloid leukaemia (AML) is a malignant disease of haematopoietic cells characterised by the accumulation of a clonal population of abnormal myeloid cells in the bone marrow (BM). The defining feature of AML is an increase in myeloid blast cells, which have failed to mature into normal haematopoietic cells due to a block in differentiation. These blast cells expand within the BM, frequently resulting in failure of normal haematopoiesis and spillage of leukaemic cells into the peripheral blood (PB) leading to a leukocytosis. AML affects patients of all ages with an overall annual incidence of 3.5 per 100,000. The incidence increases with age, and consequently the majority of cases of AML occur in elderly patients (>60 years) with a median age of presentation of 65 years. One of the most striking features of AML is the heterogeneity of this disease both in terms of the disease biology and clinical presentation. It may, therefore, be more accurate to describe AML as a group of related disorders rather than a single disease entity. The study of clinical and biological features of large cohorts of patients has allowed different subtypes of AML to be more clearly defined, with important clinical implications.

The work presented in this thesis addresses the clinical and biological implications of different types of mutation of the fms-like tyrosine kinase 3 (FLT3) gene in AML. In order to understand this work in an appropriate context, this chapter will provide an overview of the demographics, diagnosis, treatment, prognosis and the cellular and molecular pathogenesis of AML. Discussion of the specific biology and clinical relevance of FLT3 mutations is provided in the introductions to Chapters 3 to 7.

1.1 Clinical presentation of AML

The clinical features of AML at presentation are diverse and relate to the consequences of PB cytopenias or direct leukaemic cell infiltration. Patients may present with lethargy, bleeding or infection relating to anaemia, thrombocytopenia and neutropenia respectively. Leukaemic infiltration may occur in any organ, but most commonly affects the spleen, liver, gums, skin or central nervous system, leading to a wide variety of possible symptoms or clinical signs. There is also a variable rapidity of disease onset, with some patients presenting with a relatively indolent disease over many months and

others presenting with a very rapidly progressive disease with hyperleukocytosis and consequent leukostasis with neurological and pulmonary consequences. These various clinical manifestations are likely to reflect the heterogeneity of AML at a biological level.

1.2 Diagnosis and classification of AML

Historically, the diagnosis of AML was based purely on the appearance of the leukaemic blast cells in the PB or BM under light microscopy using a variety of different cytochemical stains. Current classification systems use the presence of over 20% leukaemic blast cells in the bone marrow as the defining feature of AML. The initial diagnosis of AML is still largely dependent on morphology and cytochemistry, although flow cytometry, cytogenetic and molecular techniques are becoming increasingly important in sub-classification. These different techniques have formed the basis of different classification systems to stratify cases of AML into defined subtypes.

1.2.1 French American British (FAB) classification

At a morphological level, the heterogeneity of AML is evidenced by a marked variation in the degree and lineage type of haematopoietic differentiation seen. Since 1976, AML has been classified according to the criteria of the FAB group which defines subgroups on the basis of the morphological appearance in combination with cytochemical stains and, to a certain extent, immunological methods (Bennett *et al*, 1976, Bennett *et al*, 1985). The FAB system of classification of AML is shown in Table 1.1.

1.2.2 Secondary and therapy related AML

Whilst the majority of cases of AML occur sporadically, some arise on a background of a previous bone marrow disorder such as myelodysplasia (MDS) or a myeloproliferative disease (MPD), these cases are known as secondary AML (sAML), as opposed to *de novo* AML. A third group arise as a result of previous chemotherapy or radiotherapy and these cases are known as therapy related AML (tAML). The FAB classification system takes no account of *de novo*, sAML or tAML.

1.2.3 Recurrent cytogenetic abnormalities in AML

Following the development of the FAB classification of AML, metaphase cytogenetic analysis of leukaemic blasts led to recognition of a number of recurrent cytogenetic

abnormalities in a substantial proportion of patients with AML (Table 1.2). Using conventional cytogenetic analysis, approximately 50% of patients have an abnormal karyotype (Grimwade *et al*, 1998). These abnormalities often correlate with specific morphological subgroups and, as discussed below, have become an essential component of AML classification, as the presence of some abnormalities can lead to modification of therapy. There has consequently been a move towards classification systems that incorporate these cytogenetic abnormalities. In view of the importance of the detection of some of these abnormalities, reverse transcriptase polymerase chain reaction (RT-PCR) and fluorescent *in situ* hybridization are now routinely used to improve the rates of detection of the t(8;21) (*AML1/ETO*, AML1 and eight twenty-one), inv(16) (*CBF β /MYH11*, core binding factor beta/myosin heavy chain 11) and t(15;17) (*PML/RAR α* , promyelocytic leukaemia and retinoic acid receptor α) fusion genes above that of conventional cytogenetic analysis alone (Grimwade *et al*, 1997, Langabeer *et al*, 1997a, Langabeer *et al*, 1997b).

1.2.4 World health organisation (WHO) classification

The WHO classification has updated and largely superseded the FAB classification system through a number of important modifications (Harris *et al*, 1999). The blast percentage required for the diagnosis of AML is now defined as 20%, as opposed to the previous cut-off of 30%. Some non-random cytogenetic abnormalities have been included as separate subtypes of AML, irrespective of the morphological appearance or blast percentage. Other changes include separate categories for patients with sAML, tAML, associated multilineage dysplasia and novel morphologic categories. The full WHO classification is shown in Table 1.3. These changes represent an attempt to define specific biological and clinical entities within AML by correlating clinical, morphological, immunological and genetic information. These approaches are at a relatively early stage and it is likely that such classification systems will be continuously refined as additional information on other molecular genetic information is accumulated and incorporated into them (see section 1.3.4).

1.3 Prognostic factors in patients with AML

The majority of cases of AML occur in patients greater than 60 years of age, and in this group of patients the prognosis is dismal, with 5-year overall survival (OS) rates generally less than 10%. Furthermore, there has been little improvement in outcome for

Table 1.1: The French-American-British (FAB) classification of AML

FAB subtype	Name	Cytochemistry			Immunology
		MPO	SB	NSE	
M0	AML with minimal differentiation	-	-	-	Cells are positive for MPO or other myeloid markers by flow cytometry
M1	AML without maturation	+	+	-	N/A
M2	AML with maturation	+	+	-	N/A
M3	Acute promyelocytic leukaemia	+	+	-	N/A
M4	Acute myelomonocytic leukaemia	+	+	+	N/A
M4 (Eo)	Acute myelomonocytic leukaemia with abnormal eosinophils	+	+	+	N/A
M5	Acute monocytic leukaemia	-	-	+	N/A
M6	Erythroleukaemia	+	+	-	N/A
M7	Acute megakaryocytic leukaemia	-	-	+	Cells express CD41, CD42b or CD61

MPO indicates myeloperoxidase; SB, sudan black; NSE, non-specific esterase; N/A, not applicable.

Table 1.2: Recurrent, non-random cytogenetic abnormalities in AML, underlying molecular defect and correlation with morphology

Cytogenetic abnormality	Molecular defect	Incidence in the UK MRC AML10 trial	Morphology
t(15;17)	PML-RAR α fusion gene	12%	Acute promyelocytic leukaemia (M3)
+ 8	Unknown	9%	Various
t(8;21)	AML1-ETO fusion gene	8%	AML with differentiation (M2)
inv(16)	CBF β -MYH11 fusion gene	4%	Acute myelomonocytic leukaemia with abnormal eosinophils (M4Eo) and AML with differentiation (M2)
11q23 abnormalities	Various MLL fusion genes	4%	Various
-7	Unknown	4%	Various
+21	Unknown	3%	Various
abn(3q)	Unknown	3%	Various
del(7q)	Unknown	2%	Various
del(5q)	Unknown	2%	Various
+22	Unknown	1%	Various
t(6;9)	DEK-CAN	<1%	Acute myeloblastic leukaemia with abnormal basophils

Table 1.3: The WHO classification of AML

AMLs with recurrent cytogenetic translocations
AML with t(8;21)(q22;q22), AML1(CBF-alpha)/ETO
Acute promyelocytic leukaemia (AML with t(15;17)(q22;q11-12) and variants, PML/RAR-alpha)
AML with abnormal bone marrow eosinophils (inv(16)(p13q22) or t(16;16)(p13;q11), CBFb/MYH11)
AML with 11q23 (MLL) abnormalities
AML with multilineage dysplasia
With prior myelodysplastic syndrome
Without prior myelodysplastic syndrome
AML and myelodysplastic syndromes, therapy-related
Alkylating agent-related
Epipodophyllotoxin-related (some may be lymphoid)
Other types
AML not otherwise categorized
AML minimally differentiated
AML without maturation
AML with maturation
Acute myelomonocytic leukaemia
Acute monocytic leukaemia
Acute erythroid leukaemia
Acute megakaryocytic leukaemia
Acute basophilic leukaemia
Acute panmyelosis with myelofibrosis
Acute biphenotypic leukaemias

this group of patients using conventional cytotoxic agents over the last few decades (Burnett and Mohite 2006). This adverse prognosis relates to a combination of innate drug resistance of the leukaemic cells combined with frequent co-morbidities.

Improving outcome for elderly patients is therefore a major challenge. However, in view of the very poor outcome associated with older age alone, the identification of further prognostic factors is unlikely to be helpful in the vast majority of cases. The only exception to this would be the rare cases of elderly patients who have favourable cytogenetics (see below) which may allow the identification of patients who may be more likely to respond well to therapy.

In contrast to the lack of improvement of outcome for elderly patients with AML, treatment in young adults with AML (age 18 to 60 years) has improved considerably over the last few decades such that 80% to 90% of young adults currently diagnosed with AML will enter complete remission (CR, defined as a normocellular BM aspirate

with normal trilineage maturation and less than 5% blasts) with conventional chemotherapeutic approaches (Burnett 2002a, Hann *et al*, 1997). Despite this success, however, over half of the patients who enter CR will suffer a relapse of their disease. Consequently, only approximately 50% of young adults diagnosed with AML will achieve long-term disease free survival (DFS). In view of the clinical and biological heterogeneity of AML, there has been considerable interest in identifying prognostic factors that may be useful in order to risk stratify patients and modify therapy accordingly. As the toxicity of conventional therapy in AML is considerable, it is possible that outcome could be improved by reducing the treatment intensity for patients with a favourable prognosis. Conversely, for patients with a predicted high relapse rate (RR), the intensity of treatment could be increased, or more experimental approaches used. This concept forms the basis of 'risk-adapted therapy' whereby the intensity of treatment is tailored to the specific prognosis of an individual case of AML. This thesis is focused on the group of young adult (<60 years) patients with AML and the following discussion of disease biology, prognostic factors and treatment in this chapter will be restricted to this group of patients.

1.3.1 Clinical Prognostic Factors

Analysis of the UK Medical Research Council (MRC) AML 10 and 12 trials demonstrated that the percentage of blasts in the BM after the first course of chemotherapy is an important prognostic factor (Wheatley *et al*, 1999). For example, in the UK MRC AML 12 trial, patients with >15% blasts in the bone marrow after the first course of chemotherapy have a poor prognosis (OS at 5 years 26%) compared to patients with 5-15% blasts (OS 44%) or <5% blasts (OS 56%). Other clinical prognostic factors predicting adverse outcome include increasing age, male sex, secondary AML and high white blood cell count (WBC) at diagnosis (Wheatley *et al*, 1999).

1.3.2 Cytogenetic Risk Groups

Given the frequency of recurrent cytogenetic abnormalities in AML, the prognostic implications of these abnormalities was of considerable interest but required large cohorts of patients for statistically robust conclusions to be drawn. The UK MRC AML trials allowed such analysis and showed that presentation cytogenetics is a major prognostic factor in AML (Grimwade *et al*, 1998). Consequently, patients are now routinely classified into favourable, intermediate or adverse cytogenetic risk groups. Favourable genetic abnormalities are t(8;21), inv(16), t(15;17). Patients with acute

promyelocytic leukaemia (APL) associated with the t(15;17) translocation have a particularly favourable prognosis and are now widely regarded as a biologically and clinically separate entity to non-APL AML. These patients are now treated with different protocols, including all-trans retinoic acid (Sanz 2006). Adverse genetic abnormalities are -5, -7, del(5q), abn (3q), t(9;22) or complex karyotype (five or more abnormalities). Intermediate risk karyotypes constitute all other patients not in the favourable or adverse risk groups.

In the UK MRC AML 10 trial, of the 83% of patients with karyotype data available, 24% showed translocations associated with a good prognosis, 59% fell into the intermediate and 17% into the adverse cytogenetic category. In the UK MRC AML12 trial, the 5-year overall survival of patients in each of these three groups was 73% for patients with a favourable karyotype, 44% for those with intermediate cytogenetics and 17% in the adverse cytogenetic group. If other cytogenetic abnormalities occur in addition to one of the favourable cytogenetic markers, these patients still fall into the favourable cytogenetic category. A major limitation of cytogenetic risk classification, however, is that a large proportion (approximately 50%) of patients with non-APL AML fall into an intermediate-risk cytogenetic group and karyotype is therefore not informative.

1.3.3 Combining different prognostic factors

Using a combination of cytogenetics and response to the first induction chemotherapy course, a prognostic index has been devised for use in risk stratification in the MRC AML trials (Table 1.4) (Wheatley *et al*, 1999). Essentially, this prognostic index changes the status of intermediate cytogenetic patients with >15% blasts after course 1 into a poor prognostic group. The 5-year OS in the AML 12 trial in these risk groups was 76%, 48% and 21% in the good, standard and poor risk groups respectively with RR of 25%, 52% and 73%.

This prognostic index has been updated as additional data has been acquired through the MRC trials. The new index planned for use in the UK MRC AML17 trial takes into account patient age, sex, de novo versus secondary AML, cytogenetic risk group, white blood cell count at diagnosis and BM blast percentage following cycle 1.

Table 1.4: Prognostic risk groups in AML

Good risk	Any patient with favourable genetic abnormalities t(8;21), inv(16), t(15;17), irrespective of other genetic abnormalities or marrow status after course 1
Standard	Any patient not in either good or poor risk groups
Poor risk	Any patient with more than 15% blasts in the bone marrow after course 1 or with adverse genetic Abnormalities: -5, -7, del(5q), abn (3q), t(9;22) or complex (five or more abnormalities) and without favourable genetic abnormalities

1.3.4 Molecular Prognostic Factors

Given the limitations of conventional cytogenetics for prognostic stratification in AML, one of the key questions when a novel molecular abnormality is identified in AML is whether it is associated with a specific prognostic impact, particularly in the crucial group of patients with intermediate cytogenetics. Over the last few years, a number of these molecular markers have emerged as candidate additional prognostic factors. The most prominent of these, mutations of FLT3 are the subject of this thesis and discussed in more detail in Chapters 3, 4 and 5. However, other markers have been identified. For example, nucleophosmin-1 (NPM1) gene mutations are associated with a relatively favourable prognosis in patients with a normal karyotype (NK) (Falini *et al*, 2007, Gale *et al*, 2007). CCAAT/enhancer binding protein- α (CEBPA) gene mutations may also be associated with a relatively favourable prognosis in this group (Frohling *et al*, 2004, Preudhomme *et al*, 2002). Indeed, the recent update of the WHO classification system has included cases of AML with mutations of NPM1 or CEBPA as provisional entities (Swerdlow *et al*, 2008). Conversely, mixed lineage leukaemia (MLL) gene partial tandem duplications (Basecke *et al*, 2006, Dohner *et al*, 2002, Schnittger *et al*, 2000) and mutations in the Wilms Tumour-1 gene (WT1) (Paschka *et al*, 2008, Summers *et al*, 2007, Virappane *et al*, 2008) are associated with an adverse prognosis. Interest in molecular markers has not only focused on somatic mutations but also over-expression of candidate genes. For example, high levels of expression of BAALC, WT1, EVI1 and ERG have all been shown to confer an adverse prognosis in young adults with AML (Baldus *et al*, 2003, Baldus *et al*, 2006, Barjesteh van Waalwijk van Doorn-Khosrovani *et al*, 2003, Barragan *et al*, 2004, Bergmann *et al*, 1997, Marcucci *et al*, 2005, Marcucci *et al*, 2007).

With such an abundance of different molecular markers of potential prognostic relevance, concerted efforts are required in order to determine which of these have actual clinical utility. As a number of these markers are relatively uncommon, to robustly determine their prognostic significance in AML is challenging, and this is complicated by the need to demonstrate that a marker acts independently of other known prognostic factors in a multivariate analysis. Furthermore, in order to justify incorporation of a prognostic marker into a prospective, risk-adapted therapy approach to the treatment of AML, it is necessary to demonstrate that modification of therapy on the basis of the detection of one of these markers improves outcome for patients. Therefore, in order to translate this wealth of molecular information into actual benefit for patients, the challenge is to gather sufficient information from very large cohorts with long follow up, availability of diagnostic material for analysis and sufficient numbers of patients in different treatment groups to allow statistically sound comparisons.

1.4 Conventional Chemotherapeutic Approaches in AML

Whilst there has been a gradual improvement in outcome for young adults with AML over the last 3 decades, this has occurred despite the use of very similar therapeutic agents during this period. The key agents in the management of AML are cytarabine and anthracyclines, both of which are cell-cycle dependent. It is likely that much of this improved outcome relates to better standards of supportive care allowing the use of more intensive chemotherapy with a lower risk of treatment-related mortality (TRM) (Burnett 2002a). Therapy of young adults is generally divided into two phases, remission induction therapy followed by consolidation therapy.

1.4.1 Induction Chemotherapy for non-APL AML

The goal of induction therapy is to achieve a CR with recovery of normal haematopoiesis. For the UK MRC AML 10 and 12 trials, this was defined as a normocellular BM aspirate with normal trilineage maturation and less than 5% blasts without a requirement for regeneration of peripheral counts. In practice, 97% of patients who achieved the protocol prescribed definition of CR had peripheral regeneration of neutrophils to $1.0 \times 10^9/\text{L}$ and platelets to $100 \times 10^9/\text{L}$. Standard induction chemotherapy in AML consists of cytarabine in combination with anthracyclines or

purine analogues. The use of different anthracyclines has not consistently affected outcome (Milligan *et al*, 2006). Using this approach, up to 80% to 90% of patients will enter CR. Details of the induction regimens used in the MRC AML 10 and 12 trials are given in Chapter 4.

1.4.2 Consolidation Chemotherapy

Once a patient has achieved CR, further treatment to consolidate this remission is necessary as, due to the presence of residual leukaemic cells, the median DFS for patients who receive no additional therapy is only 4-8 months (Cassileth *et al*, 1998). High dose cytarabine is widely accepted to be the consolidation chemotherapy of choice in AML. The number of courses, dose of cytarabine and combinations with other agents, however, remains uncertain (Milligan *et al*, 2006). Whether a total of 4 or 5 courses of chemotherapy are required is also uncertain. Details of the induction regimens used in the MRC AML 10 and 12 trials are given in Chapter 4.

1.4.3 Autologous stem cell transplantation (SCT)

The high RR associated with patients with non-APL AML who enter CR led to enthusiasm for the use of high dose therapy, usually a combination of total body irradiation and cyclophosphamide, followed by autologous transplantation with stem cell support, thereby allowing higher doses of chemotherapy or radiotherapy to be administered than could otherwise be tolerated. This approach has been shown to reduce the RR in a number of different trials. However, due to the additional toxicity of the procedure, an OS advantage has not been shown. Autologous transplantation has now therefore been largely abandoned as a consolidation therapy in current clinical trials.

1.4.4 Allogeneic SCT

Myeloablative, allogeneic SCT from a sibling or unrelated matched donor combines high dose chemotherapy with an additional graft versus leukaemia (GvL) effect. Most clinical trials have shown a clear benefit in terms of a reduction in RR using this approach. However the OS benefit, if any, is modest due to the increased toxicity associated with the procedure (Burnett 2002b) with TRM of between 15-35% in most clinical trials. Furthermore, the toxicity of this procedure increases with age and therefore myeloablative allogeneic SCT is generally restricted to adults less than 40 years of age. By reducing the intensity of conditioning therapy prior to allogeneic SCT (RIC-SCT) the GvL effect can be harnessed without the toxicity associated with

myeloablative regimes. Preliminary data suggests that RIC-SCTs are a promising new treatment in AML, worthy of further evaluation in ongoing clinical trials (Tauro *et al*, 2005, Taussig *et al*, 2003).

1.4.5 Acute Promyelocytic Leukaemia (APL)

It is now widely accepted that APL is a biologically and clinically distinct disease with specific diagnostic criteria and treatment protocols that are markedly different from those of non-APL AML (Sanz 2006). As discussed above, the outcome for this group of patients using modern treatment approaches is favourable compared to non-APL AML. It is therefore extremely important to identify patients with APL using molecular techniques as well as conventional cytogenetics in order that they can be treated appropriately. Combination of all-trans retinoic acid (ATRA) with standard chemotherapy regimes during induction and/or consolidation results in high rates of CR and OS (70-75% at 5 years) in APL. It is not clear if high doses of myelosuppressive drugs are required to achieve such high rates of DFS as a Spanish trial group have shown that a combination of anthracycline and ATRA without other myelosuppressive drugs can achieve similar rates of remission (Sanz *et al*, 2004). A comparison of the two approaches was the purpose of the MRC AML 15 trial for patients with APL. This randomisation is now closed and the current recommendation is that patients are treated according to the Spanish protocol.

1.5 Risk-adapted therapy

A limited degree of risk-adapted therapy has been introduced into current AML treatment using the above prognostic factors (Table 1.4), although different approaches have been adopted by different groups. For example, the UK MRC approach is to exclude good risk patients from receiving an allogeneic SCT. Standard risk patients receive an allogeneic SCT if they have a matched sibling donor available and poor risk patients should receive a sibling or matched unrelated donor allogeneic SCT if a matched donor can be found. This approach is supported by a recent meta-analysis which used a 'genetic randomisation' to demonstrate that patients with favourable cytogenetics did not benefit from availability of a sibling donor, as opposed to those with non-favourable cytogenetics, i.e. standard and poor risk patients who had a survival advantage if a sibling donor was available (Cornelissen *et al*, 2007). In this

analysis, patients greater than 35 years did not benefit from the availability of a donor, although these data were accrued largely before the advent of RIC-SCTs.

A major limitation of current risk stratification strategies is demonstrated by this analysis as only 15% of patients fell into the favourable cytogenetic category and 55% had intermediate risk cytogenetics, i.e. in the majority of patients, cytogenetics is not informative with regards to prognosis. This highlights the unmet need for additional prognostic factors that will help to stratify these heterogeneous intermediate risk patients into good and poor prognostic groups so that their therapy can be tailored appropriately.

1.5.1 Monitoring for minimal residual disease (MRD)

As relapse is the major cause of treatment failure in AML, the identification of markers for the detection of MRD in patients who are in morphological CR may also provide important prognostic information and allow a degree of response-adapted therapy. This approach has been used with a great deal of success in paediatric acute lymphoblastic leukaemia (ALL) treatment protocols (Coustan-Smith *et al*, 2000). There is also increasing evidence that monitoring for MRD in APL reliably identifies patients who would relapse in the absence of additional therapy (Lo-Coco *et al*, 2003). Whilst there is some evidence that earlier treatment at molecular rather than haematological relapse in APL may improve outcome (Esteve *et al*, 2007), this data remains controversial. At present, however, monitoring for MRD in non-APL AML remains an experimental tool for use in the context of clinical trials. It is important to recognise, however, that for any given MRD marker to be of clinical use the marker must be stable through the course of the disease i.e. present both at diagnosis and relapse.

1.6 Molecular and cellular pathogenesis of AML

Understanding the biology of malignant diseases at the cellular and molecular level is essential to refine diagnostic and/or classification strategies, improve risk stratification approaches, detect novel markers for MRD analysis and develop novel therapies. In view of this, major efforts have been made over the last 2 decades to identify genes that are mutated in malignancies, including AML. A key group of genes that are dysregulated in cancers are known as oncogenes.

1.6.1 Oncogenes

An oncogene can broadly be defined as a protein encoding gene which, when deregulated, participates in the onset and development of cancer. The landmark description of oncogene-induced cancer growth was made through the study of Rous Sarcoma Virus-transformed cells (Martin 1970). The subsequent characterisation of the retroviral oncogene v-src and its normal cellular counterpart c-src by J. Michael Bishop and Harold E. Varmus led to the award of the Nobel Prize in Medicine in 1989 (Marx 1989). Oncogenes encode proteins that are involved at any stage of the control of cell cycle and/or apoptosis regulation, thus dysregulation leads to increased cell proliferation and/or survival signals. They can be categorised into six main groups:

- Growth factors e.g. *INT2* in breast cancer
- Growth factor receptors (GFRs) e.g. *FLT3* in AML
- Signal transduction proteins e.g. *H-RAS* in colon cancer
- Transcription factors e.g. *MYC* in Burkitt lymphoma
- Chromatin remodelers e.g. *MLL* in AML or ALL
- Apoptosis regulators e.g. *BCL2* in lymphomas

Dysregulation of oncogenes in human malignant diseases usually occurs through somatic genetic events or mutations, although it may also occur on the background of a germ-line genetic predisposition to cancer or through epigenetic events (Croce 2008). Early evidence that human cancer arises due to somatic genetic events arose from studies of Burkitt lymphoma. It was shown that translocation of the *MYC* oncogene to enhancer regions of the immunoglobulin gene loci led to overexpression of the *MYC* protein (Croce *et al*, 1983, Dalla-Favera *et al*, 1982). Furthermore, transgenic mice overexpressing *MYC* developed fatal lymphomas with a short latency (Adams *et al*, 1985). Overexpression of oncogenes can also arise through a number of different mechanisms in addition to oncoretroviral or chromosomal translocation related events including gene amplification indirectly through mutation of a gene involved in regulation of expression of the protein. Oncogenes can also be activated by somatic mutation of the gene, abnormal expression of isoforms or gene fusion leading to constitutive activation of the protein product. An early example of an activating mutation of a human oncogene was the *H-RAS* and *K-RAS* oncogenes, as demonstrated by transfecting mouse fibroblasts with genetic material from bladder and colon cancer cell lines respectively (Capon *et al*, 1983, McCoy *et al*, 1983). When mutated in codons

12, 13 or 61, these *RAS* signal transduction genes encoded a protein that was constitutively activated, irrespective of the normal cellular control mechanisms. This, in turn, led to continuous activation of downstream signalling proteins, with the end result of an increased rate of cell proliferation and survival signalling. The recognition of the central role of mutated oncogenes in human malignancy, and their potential diagnostic and therapeutic importance, subsequently induced major efforts to identify dysregulated oncogenes in a wide range of different malignancies.

1.6.2 Mutations in growth factor receptors in AML

Despite extensive efforts to identify activated oncogenes in AML, by the mid-1990s, these remained largely unknown. Mutations in *N-RAS* and *K-RAS* had been identified, but these were relatively uncommon in AML, occurring in less than 20% of cases (Bos *et al*, 1985, Bowen *et al*, 2005). Therefore, aside from the recurrent cytogenetic translocations described above, the somatic events leading to oncogene activation in the majority of cases of AML remained unknown. As haematopoiesis is controlled by haematopoietic growth factors binding to their respective receptors, many studies attempted to identify GFRs that may be aberrantly activated in AML. Such haematopoietic GFRs fall into two main categories. Firstly, receptor tyrosine kinases (RTKs) such as c-Fms, c-Kit and FLT3 with intrinsic tyrosine kinase activity. Secondly, haemopoietin receptors such as erythropoietin receptor (EPO-R), thrombopoietin receptor, interleukin-3 receptor (IL-3R), IL-5R, granulocyte colony stimulating factor receptor (G-CSFR) and granulocyte macrophage colony stimulating factor receptor (GM-CSFR). This latter group do not have intrinsic tyrosine kinase activity and associate non-covalently with intracellular tyrosine kinases.

However, despite biological evidence of a possible role for GFR mutations in AML (Longmore and Lodish 1991), up to the mid-1990s, screening of the haemopoietin receptors GM-CSF (Decker *et al*, 1995, Freeburn *et al*, 1998), EPO-R (Le Couedic *et al*, 1996) and G-CSF (Forbes *et al*, 2002) as well as the RTKs c-Fms and c-Kit (Arland *et al*, 1994) suggested that such activating mutations were rare events in AML. However, the detection of activating mutations in the RTK FLT3 in one quarter of young adults with AML (Nakao *et al*, 1996) heralded a new era in the understanding of genetic events underlying AML pathogenesis. The subsequent characterisation of these FLT3 mutations are discussed in more detail in the introductions of Chapters 3 to 7.

1.6.3 Cooperating genetic events in leukaemogenesis

Transformation of a normal haematopoietic cell into a leukaemic cell requires not only an abnormal proliferative or survival signal, which may arise from an activated oncogene, but also an abnormal capacity for haematopoietic differentiation as well as maintenance of self-renewal capacity (Kelly and Gilliland 2002). Therefore, unless a single genetic event is sufficient to confer all of the above properties to a haematopoietic cell, a number of cooperating genetic events are necessary for the development of AML. This has been simplified as a ‘two-hit’ model of leukaemogenesis with class I mutations conferring a proliferative and/or survival advantage and class II mutations impairing haematopoietic cell differentiation (Kelly and Gilliland 2002).

Cloning of the chromosomal translocation breakpoints associated with AML has identified, in many cases, fusion genes that are directly implicated in leukaemogenesis (Kelly and Gilliland 2002). The fusion genes involve transcription factors or coactivators of transcription in the majority of cases. Some genes are involved in multiple different chromosomal translocations, e.g. the CBF complex and RAR α , which are both important in the regulation of normal haematopoiesis. Dysregulation of these genes leads to impairment of haematopoietic differentiation and/or increased self-renewal capacity but may not confer a marked proliferative advantage to a cell, i.e. they are type II mutations. Experimental evidence for this derives from a number of animal models of leukaemia where artificial expression of PML/RAR α , AML1/ETO or inv(16) alone leads to impairment of haematopoietic differentiation and expansion of the stem cell pool but not an overt leukaemic phenotype (Castilla *et al*, 1999, Grisolan *et al*, 1997, He *et al*, 1997, Higuchi *et al*, 2002). Leukaemia only developed if the animals were exposed to chemical mutagens such as n-ethyl-n-nitrosourea, suggesting that additional genetic events (type I mutations) are required for a leukaemic phenotype. Similarly, expression of an activated RAS or FLT3 in a mouse model leads to a myeloproliferative phenotype but not acute leukaemia (Kelly *et al*, 2002b, MacKenzie *et al*, 1999). Expression of both activated FLT3 and PML/RAR α leads to the development of acute leukaemia with high penetrance and short latency (Kelly *et al*, 2002a).

Further supporting evidence for the concept of collaborating genetic events in AML derives from the observation in humans that germline mutation of AML1 alone is

insufficient for the development of AML and further mutations later in life are required for the development of leukaemia (Song *et al*, 1999). This is in keeping with observations in ALL that the TEL/AML1 fusion gene is present at birth in children who went on to develop ALL later in life as assessed by polymerase chain reaction (PCR) analysis of genomic DNA (gDNA) from Guthrie cards (Wiemels *et al*, 1999). Studies in syngeneic twins have also shown that a 'pre-leukaemic' TEL/AML1 positive clone is likely to predate the development of ALL, again suggesting that a second mutation is required for development of leukaemia (Hong *et al*, 2008).

In view of these multiple lines of evidence, it is now widely accepted that the majority of cases of AML are caused by cells which have acquired a series of genetic 'hits' over time. Exactly how many 'hits' are required for leukaemia development, however, remains uncertain (Rangarajan *et al*, 2004). A particular exception to this, however, may occur with MLL fusion genes that may be sufficient alone to cause acute leukaemia (Barabe *et al*, 2007).

1.6.4 Leukaemic stem cells

Understanding the cellular as well as genetic basis of AML is also important with regards to improving diagnosis and therapy. This is because AML not only demonstrates heterogeneity between cases, but also cells within a particular leukaemic clone can have different biological characteristics. For example, an early study using tritiated thymidine incorporation in two adults with leukaemia showed that only a small proportion (5.6% and 6.1% respectively) of the blast cells in the bone marrow were actively proliferating (Clarkson *et al*, 1967). This may have important implications for the therapy of AML which mostly relies on cell cycle-dependent agents. One of the most important questions with regards to this heterogeneity within a particular population of leukaemic cells is whether all cells are capable of propagating and renewing the leukaemia. It has become increasingly apparent that this is not the case and that leukaemic cells form a hierarchy, in a similar way to normal tissues, with stem cells giving rise to progenitor cells, in turn giving rise to more mature, blast cells. Consequently, only a certain fraction of the leukaemic cells, leukaemic stem cells (LSCs), are capable of renewing the complete leukaemic hierarchy. These LSCs are likely to be similar to the target cell of transformation and also, importantly, in patients who enter CR, LSCs are likely to be the cells that are responsible for relapse. Identifying the cellular phenotype of these cells may therefore allow their specific

targeting with therapeutic agents. The definitive studies with regards to this used non-obese diabetic mice with severe combined immunodeficiency (NOD-SCID) to demonstrate that only approximately 1 in 10^6 cells within a leukaemic population are capable of initiating a leukaemia in this transplantation model (Bonnet and Dick 1997). Using fluorescence activated cell sorting (FACS) these studies demonstrated that these NOD-SCID leukaemia initiating cells (SL-ICs) have a CD34⁺CD38⁻ cell surface phenotype. These SL-ICs share a number of biological features associated with stem cells in that they have extensive proliferative and self-renewal capacity but differ in that they have an impaired capacity to differentiate into normal, mature haematopoietic cells. There is, however, still considerable uncertainty as to whether these LSCs are formed by damage to normal haematopoietic stem cells (HSCs) or whether haematopoietic progenitor cells (HPCs) acquire the genetic hits that therefore confer stem cell-like properties. There is some evidence for the latter in mouse models (Kirstetter *et al*, 2008, Krivtsov *et al*, 2006) but this needs to be recapitulated in human model systems. The frequency of LSCs is also controversial with some mouse models suggesting that LSCs are relatively frequent within the leukaemic hierarchy (Kelly *et al*, 2007, Krivtsov *et al*, 2006, Somervaille and Cleary 2006) and that the apparent very low frequency of SL-ICs in previous studies may have been due to inefficiencies of xenotransplantation.

Despite the controversy with regards to the frequency of LSC, there is wide acceptance that these LSCs exist and that they have a very high self-renewal capacity combined with impaired differentiation capability. Due to their lack of proliferative capacity, current cell-cycle dependent therapeutic strategies are unlikely to target this type of cell and therefore the current challenge is to identify novel therapeutic agents that may selectively target these LSCs. In order to achieve this, it is essential to understand how individual oncogenes disrupt the process of differentiation, proliferation and self-renewal in leukaemia.

1.7 Novel Therapeutic Approaches in AML

The importance of different cytogenetic abnormalities in predicting the response to therapy suggests that an increased understanding of the underlying molecular defects in AML may in turn provide insights into the mechanisms of chemoresistance in AML. Furthermore, the identification of some of the molecular defects underlying recurrent

cytogenetic abnormalities may have more direct therapeutic benefits. For example, the identification of the *PML-RARα* fusion gene in over 95% of cases of APL with t(15;17) led to the use of ATRA in this disorder with profound therapeutic benefits (Lo-Coco and Ammatuna 2006, Mistry *et al*, 2003). *RARα* is a member of the RA nuclear receptor family and is a ligand-inducible transcriptional activator through histone acetylation. The PML-RARα fusion protein acts as an aberrant receptor leading to altered DNA-binding affinities and repression of normal RA signalling. This leads to the differentiation block in haematopoiesis that characterises APL. Administration of supra-physiological levels of ATRA leads to a conformational change in the PML-RARα fusion protein and consequent activation of previously repressed RARα target genes. Furthermore, ATRA induces the degradation of the PML-RARα fusion protein through the ubiquitin/proteasome system. Thus, ATRA is directly targeting the underlying molecular defect in AML. The dramatically improved rates of DFS using ATRA-based therapeutic approaches in APL has led to enthusiasm for the development of other such therapeutic approaches targeting other recurrent molecular defects in AML.

1.7.1 Targeted inhibitors

Oncogenic proteins in cancer cells can be targeted using small molecules which specifically inhibit an activated oncogene. The remarkable success of imatinib in the treatment of chronic myeloid leukaemia (CML) is the paradigm for this approach, and this is discussed in more detail in Chapter 6.

1.7.2 Other novel therapies

Whilst the addition of higher doses or more courses of conventional chemotherapy is effective at reducing the risk of relapse in AML, this has to be balanced against the additional toxicity that arises as a result. Monoclonal antibody therapy is an alternative to conventional cytotoxic agents in that the specificity of the antibody allows the leukaemic cells to be targeted whilst minimising toxicity. A humanised anti-CD33 monoclonal antibody conjugated to calicheamicin has demonstrated considerable efficacy in the treatment of high-risk relapsed or refractory patients with AML and is currently being assessed in large phase 3 clinical trials (Pagano *et al*, 2007). Other approaches aim to modify drug resistance mechanisms, epigenetic changes, angiogenesis and apoptotic mechanisms (Stone 2007).

1.8 Aims of this thesis

FLT3 mutations in AML are heterogeneous with 2 main classes of mutation, internal tandem duplications (ITDs) and tyrosine kinase domain (TKD) mutations. The objective of the studies described in this thesis was to compare and contrast the incidence (Chapter 3), clinical implications (Chapters 4 and 5) and biological impact (Chapter 7) of these different types of mutation in AML as well as the impact of FLT3 inhibitors according to FLT3 mutations status (Chapter 6).

CHAPTER 2: MATERIALS AND METHODS

Specific methods are discussed in the relevant results chapter (Chapters 3-7).

2.1 Cell Culture

2.1.1 Cell lines

- 293T human embryonal kidney line, adherent, growth factor independent
- 32Dcl3 murine myeloid line, non-adherent, growth factor dependent
- Ba/F3 murine pro-B line, non-adherent, growth factor dependent
- HeLa human cervical carcinoma line, adherent, growth factor independent
- NB4 human promyelocytic leukaemia line, non-adherent, growth factor independent

2.1.2 Cell culture general reagents

- 0.25% Trypsin-EDTA (Invitrogen, Paisley, UK)
- 0.4% Trypan Blue (Sigma Aldrich, Poole, UK)
- Antibiotic-Antimycotic Solution (Sigma Aldrich, Poole, UK)
- Calibration beads for flow cytometry (Dako, Glostrup, Denmark)
- Dimethyl sulfoxide (Sigma Aldrich, Poole, UK)
- Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Paisley, UK)
- Ficoll-Paque™ gradient (Amersham Biosciences, Bucks, UK)
- Foetal calf serum (FCS), heat inactivated for 30 minutes at 56°C (PAA Laboratories, Pasching, Austria)
- MACS® Cell Separation Columns and Reagents (Miltenyi Biotec, Germany)
- Methocult™ (Stem Cell Technologies, Vancouver, Canada)
- MTS tetrazolium compound (Promega, Southampton, UK)
- Phosphate buffered solution (PBS) (Invitrogen, Paisley, UK)
- Polybrene (Sigma Aldrich, Poole, UK)
- Propidium iodide (Invitrogen, Paisley, UK)
- RPMI-1640 medium with L-glutamine (Invitrogen, Paisley, UK)
- StemPro-34 (Invitrogen, Paisley, UK)

- X-vivo10 medium (Cambrex, Bath, UK)

2.1.3 Tyrosine Kinase Inhibitors

- Lestaurtinib (CEP701) (Cephalon, West Chester, PA, USA)
- PKC412 (Novartis, Basel, Switzerland)

2.1.4 Other therapeutic agents

- All-trans retinoic acid (ATRA) (Sigma Aldrich, Poole, UK)
- Cytarabine arabinoside (Ara-C) (David Bull Laboratories, Victoria, Australia)

2.1.5 Recombinant growth factors

- Human G-CSF (PeproTech, London, UK)
- Human FLT3-ligand (PeproTech, London, UK)
- Human IL3 (PeproTech, London, UK)
- Human thrombopoietin (PeproTech, London, UK)
- Murine IL-3 (PeproTech, London, UK)
- Human SCF (PeproTech, London, UK)

2.1.6 Cell culture plastics

- 80 cm² and 175 cm² flasks (Nalge Nuc, Rochester, NY, USA)
- 6, 12, 24 and 96 well tissue culture plates (Nalge Nunc, Rochester, NY, USA)

2.1.7 Ficoll-Hypaque centrifugation

Cells were washed in RPMI containing 2% FCS (R2) and resuspended in R2 at a density of $10\text{--}20 \times 10^6/\text{L}$. Cells were then layered onto an equal volume of Ficoll-Paque™Plus (Amersham, Bucks, UK), and centrifuged at 800g for 20 minutes. Cells from the interface were removed, washed twice in R2 and resuspended to the required concentration in R10 (RPMI-1640 containing 10%FCS).

2.1.8 Cell cryopreservation and thawing

Primary cells and cell lines in R10 were frozen at a density of $10 \times 10^6/\text{mL}$ by adding an equal volume of freezing mix (RPMI [30%], FCS [50%] and DMSO [20%]) and stored in liquid nitrogen until required. Cells were thawed at room temperature and very slowly resuspended in 10mLs of R10 by the addition of small volumes of pre-warmed

R10 with regular and thorough mixing. Then, cells were pelleted and washed in R10 and viability assessed by trypan blue exclusion. If the viability was less than 90%, viable cells were selected by Ficoll-Hypaque centrifugation.

2.1.9 Trypan blue exclusion

Trypan blue exclusion was used to determine the density of viable cells in a cell suspension. It is based on the principle that viable cells have an intact cell membrane and are therefore able to exclude the trypan blue, unlike non-viable cells. Viable cells therefore can be identified as they retain a clear cytoplasm, unlike non-viable cells which have a blue cytoplasm when visualised by light microscopy. The cell suspension to be tested was mixed with an equal volume of 0.4% trypan blue and incubated at room temperature for 2-5 minutes. The trypan blue cell suspension was then transferred to a haemocytometer and the number of viable and non-viable cells counted under a light microscope.

2.1.10 Propidium iodide staining

Propidium iodide (PI) is a DNA intercalating agent and a fluorescent molecule that can be used to stain DNA in order to differentiate between necrotic, apoptotic and normal cells. When bound to nucleic acids, the absorption maximum for PI is at 535 nm and the fluorescence emission maximum is 617 nm. As PI is not able to cross the cell membrane, it is excluded from viable cells and therefore the fluorescence of viable cells is lower than that of dead cells. Cells were incubated for 5 minutes at 4°C with 2µg/mL of PI and then analysed on a flow cytometer.

2.2 Molecular Biology

2.2.1 Reagents

- γ -[³²P]-ATP (3000 Ci/mmol, Amersham Pharmacia Biotech, Little Chalfont, UK)
- 3MM Chr chromatography paper (Sigma Aldrich, Poole, UK)
- Acetonitrile (Transgenomic Ltd, Glasgow, UK)
- Acrylamide/NN'methylene bis-acrylamide 30%:0.8% (National Diagnostics, Hull, UK)
- Agar (Calbiochem, UK)
- Agarose (Sigma Aldrich, Poole, UK)

- Ammonium persulphate (APS) (Sigma Aldrich, Poole, UK)
- Bioline buffer and magnesium chloride (Bioline, London, UK)
- BIOTAQ™ polymerase (Bioline, London, UK)
- Bromophenol blue (VWR International Ltd, Lutterworth, UK)
- Carbenicillin (Invitrogen, Paisley, UK)
- Chloroform (VWR International Ltd, Lutterworth, UK)
- DH5α competent bacteria (Invitrogen, Paisley, UK)
- DNA size standard kit – 400 (Beckman Coulter UK Ltd., Buckinghamshire, UK)
- dNTPs (Bioline, London, UK)
- DTAB (dodecyl-trimethyl ammonium bromide) (Sigma Aldrich, Poole, UK)
- DTCS Quick Start kit (Beckman Coulter UK Ltd., Buckinghamshire, UK)
- EDTA (ethylenediaminetetraacetic acid disodium salt) (Sigma Aldrich, Poole, UK)
- Ethanol 100% (VWR International Ltd, Lutterworth, UK)
- Ethidium Bromide (Invitrogen, Paisley, UK)
- Glycerol (VWR International Ltd, Lutterworth, UK)
- HEPES (Sigma Aldrich, Poole, UK)
- IPTG (Sigma Aldrich, Poole, UK)
- KCl (VWR International Ltd, UK)
- Luria-Bertani (LB) broth (Anachem, Luton, UK)
- Low melting point agarose (Invitrogen, Paisley, UK)
- N’N’N’N-tetra-methylethylenediamine (TEMED) (Biorad, CA, USA)
- Na acetate (VWR International Ltd, Lutterworth, UK)
- NaCl (VWR International Ltd, Lutterworth, UK)
- Optimase buffer and magnesium sulphate (Transgenomic Ltd, Glasgow, UK)
- Optimase™ polymerase (Transgenomic Ltd, Glasgow, UK)
- Orthoboric acid (VWR International Ltd, Lutterworth, UK)
- Primers (Invitrogen, Paisley, UK)
- Promega T4 DNA Ligase (Promega, Southampton, UK)
- QIAGEN Plasmid Mini- and Mega-prep kits (Qiagen, Crawley, UK)
- QIAquick Gel Extraction Kit (Qiagen, Crawley, West Sussex, UK)
- QIAquick PCR Purification kit (Qiagen, Crawley, UK)
- Restriction enzymes and buffers (New England Biolabs, Hitchin, UK)

- Sample loading solution (SLS) (Beckman Coulter UK Ltd., Buckinghamshire, UK)
- SDS (sodium dodecyl sulphate) (Sigma Aldrich, Poole, UK)
- SOC medium (Invitrogen, Paisley, UK)
- T4 polynucleotide kinase (Promega, Southampton, UK)
- TOPO TA Cloning Kit (Invitrogen, Paisley, UK)
- Tri-ethylene ammonium acetate (TEAA) (Transgenomic Ltd, Glasgow, UK)
- Tris (Hydroxymethyl) methylamine (VWR International Ltd, Lutterworth, UK)
- X-Gal (Sigma Aldrich, Poole, UK)

2.2.2 Buffers

2 x HBS: 50mM HEPES, pH 7.05; 10 mM KCl; 12 mM Dextrose; 280 mM NaCl; 1.5 mM Na₂HPO₄ (FW 141.96). The final pH of the solution was adjusted to 7.05 +/- 0.05. Filtered through a 0.2 µM filter, aliquoted, and stored at -20°C.

DNA lysis buffer: 20g DTAB (final concentration 8%), 22g NaCl (final concentration 1.5 M), 25 mLs of 1M Tris Cl pH 7.8 (100 mM). Made up to 250 mLs with dd H₂O (double distilled H₂O).

10x TBE (pH8.3), for 1 litre: Tris 108.9g, orthoboric acid 55.7g, EDTA 7.4g.

Loading buffer: 30% glycerol, 0.025% bromophenol blue in 1xTBE.

2.2.3 DNA Extraction

Cells were suspended in PBS at a concentration of 1×10^6 cells per 100 µL, double the volume of DNA lysis buffer added, mixed well and incubated at 68°C for 5 minutes. The sample was then cooled, mixed with an equal volume of chloroform and centrifuged at 2000g for 20 minutes. The upper layer, containing the DNA in solution, was carefully decanted, added to an equal volume of 100% ethanol and gently mixed until the DNA precipitated. The DNA was transferred to a clean tube and washed twice in 70% ethanol, then dissolved by end-over-end rotation at 4°C in an appropriate volume of ddH₂O. Samples were stored at 4°C.

2.2.4 Polymerase chain reaction (PCR)

The PCR was used to amplify specific regions of genomic and complementary DNA. The DNA is amplified by successive cycles of denaturation, sequence-specific forward and reverse primer annealing and DNA polymerisation. Two different Taq polymerases were used during this work, BIOTAQ™ and Optimase™, each with specific conditions as recommended by the manufacturer. For BIOTAQ™ these were: 1x reaction buffer (160mM (NH₄)₂SO₄, 67mM Tris-HCl, 0.1% Tween-20), 2mM MgCl₂, 200 µM of each dNTP, 200 nM of each forward (5') and reverse (3') oligonucleotide primer and water to the required volume prior to the addition of DNA template. In order to ensure uniformity of conditions in each reaction tube, a master mix containing the necessary reagents was made prior to the addition of DNA template or ddH₂O for the negative control. For BIOTAQ™ the cycling conditions were denaturation at 95°C for 30 seconds followed by primer-annealing step for 30 seconds (temperature dependent on the composition of the primers) and a primer extension step at 72°C for 30 seconds. These 3 steps were repeated for a variable number of cycles as specified in the specific materials and methods section of each chapter. Following this, a final extension step was carried out at 72°C for 5 minutes to ensure complete extension of all primers. For Optimase™ the conditions were as specified by the manufacturer; concentrations of dNTPs and primers were as used for BIOTAQ™. An initial denaturation step was added (95°C for 5 minutes) and the primer extension step was longer (1 minute at 72°C rather than 30 seconds).

2.2.5 Agarose gel electrophoresis

Agarose gels (1.0% - 3.5%) were used to confirm the presence of PCR products of appropriate size and purity as well as to identify bands of different sizes following restriction enzyme digestion of PCR products or plasmid DNA. The required amount of agarose was added to 35mL of 1xTBE, dissolved by heating in a microwave oven and cooled before the addition of 5 µL ethidium bromide (1mg/mL). The gel was then poured into a mould and allowed to set. Appropriate volumes of PCR product or restriction enzyme digest were mixed with loading buffer, loaded into the wells and electrophoresed in 1xTBE (containing 200ng/mL ethidium bromide) using an appropriate voltage. PCR products were visualised under a UV transilluminator and digital and/or Polaroid images were made.

2.2.6 End-labelling of primers

One primer from a given primer pair was labelled with a radioactive isotope to allow visualisation and quantification of digested bands as detailed in Chapter 5. To do this, 10pmol of primer was incubated with 10 units of T4 polynucleotide kinase in 1x reaction buffer (70mM Tris-HCl, 10mM MgCl₂, 50mM 1,4-dithiothreitol) and 3 µl γ-[³²P]-ATP (3000 Ci/mmol) for 30 minutes at 37°C which resulted in the exchange of the 5' phosphate of the primer with γ-[³²P]-ATP. Following the incubation, samples were incubated at 95°C for 5 minutes to inactivate the enzyme. The labelled primer was then used for PCR as above.

2.2.7 Polyacrylamide gel electrophoresis

Polyacrylamide gels were used for quantitative analysis of digested PCR products as detailed in Chapter 5. Denaturing polyacrylamide gels consisted of 7 M urea, 6% polyacrylamide (cross-linker ratio 37.5:1) and 1 x Tris-borate-EDTA. To catalyse acrylamide polymerisation, 50 µL TEMED and 250 µL 10% APS were added to 50mLs of gel solution immediately prior to pouring. The gel was poured into a cast of 33cm x 40cm with 0.4mm spacers and allowed to polymerise. Before loading, PCR products were mixed with loading solution (95% formamide, 20mM EDTA, 0.05% Bromophenol blue, 0.05% xylene cyanol), incubated for 5 minutes at 95°C to denature and quenched on ice. Following sample loading they were electrophoresed in 0.5xTBE at 1500V until the xylene cyanol ran about two thirds of the way down the plate (approximately 3.5 hours). The gels were transferred to 3MM Chr chromatography paper, dried and exposed to a Fuji BAS-IIIi imager plate overnight.

2.2.8 Restriction enzyme digestion

All restriction enzymes used were obtained from New England Biolabs and PCR products or plasmid DNA were digested overnight according to the manufacturers specified conditions.

2.2.9 DNA Sequencing protocol

PCR products were purified using the QIAquick PCR purification kit with the manufacturers' recommended protocol. Dye-terminator sequencing reaction was then performed using the CEQ™ DTCS Quick Start kit (a single reaction mix containing the dye terminators, DNA polymerase, MgCl₂ and reaction buffer) according to manufacturers' recommended conditions with the addition of approximately 20ng

purified PCR product and 3.2pmol of the required oligonucleotide primer. Twenty-five amplification cycles of 96°C for 20 seconds, 50°C for 20 seconds and 60°C for 4 minutes were performed. The sequencing reaction products were precipitated using 2.0µL Na acetate (3M, pH 4.6), 1 µL 20mg/mL glycogen and 50µL 95% ethanol and incubated at room temperature for 10 minutes. The DNA precipitate was pelleted by centrifugation at 15,000 g for 20 minutes. The pellet was washed twice in 75% ethanol and allowed to air dry prior to resuspension in 15µL SLS. The sample was then sequenced on a CEQ8000 Beckman Coulter DNA Genetic Analysis System.

2.2.10 LB broth and plates

LB broth was made by adding 4 LB tablets to 250mLs of ddH₂O followed by autoclaving. For LB-agar plates, 3.75g of agar was added to the above mix, again followed by autoclaving. Once cool to touch, carbenicillin (final concentration 50µg/mL) was added, if required. Plates were then poured, dried for 1 hour and stored at 4°C until required.

2.2.11 Cloning of PCR products

PCR products were cloned using the TA cloning system which utilises the polyadenosine overhang at the 3' end of PCR products generated using a non-proofreading Taq polymerase. PCR products were ligated with pCR®2.1-TOPO plasmid which contains deoxythymidine overhang at the insertion site by incubating 0.5 - 4µL of PCR product with 1µL vector and 1µL salt solution (200mM NaCl; 10mM MgCl₂) in a final volume of 6µL at room temperature for 5 minutes. The vector was then transformed into DH5α competent bacteria by heatshock at 37°C for 30 seconds and the cells were then placed on ice for 2 minutes. LB agar plates (1xLB, 15% agar, 50µg/mL carbenicillin) were pre-coated with 40µL IPTG (100 mM) and 20 µL X-Gal (50mg/mL) which were allowed to absorb for 30 minutes at 37°C. The transformed bacteria were plated and incubated overnight at 37°C. The presence of an inserted PCR sequence was detected by the presence of white colonies due to disruption of the lacZ gene in the pCR®2.1-TOPO plasmid. Colonies without an insertion were blue. Transformed colonies were picked and expanded in LB medium containing 50µg/mL carbenicillin and products for sequencing obtained either by direct PCR of bacterial cells or following preparation of plasmid DNA using the QIAGEN Plasmid Mini-prep kits.

2.3 SDS-PAGE, Immunoblotting and Flow Cytometry

2.3.1 Reagents

- Aprotinin (Sigma Aldrich, Poole, UK)
- Bovine Serum Albumin (BSA) (Sigma Aldrich, Poole, UK)
- BD-Perm/Wash™ (BD-Biosciences, Oxford, UK)
- DL-Dithiothreitol (DTT) (Sigma Aldrich, Poole, UK)
- Enhance chemiluminescence kits (ECL and ECL-plus) (Amersham Life Sciences, Bucks, UK)
- Hybond-C-Extra nitrocellulose membrane (Amersham Life Sciences, Bucks, UK)
- Hyperfilm™ high performance autoradiography film (Amersham Life Sciences, Bucks, UK)
- Leupeptin (Sigma Aldrich, Poole, UK)
- Microcystin LR (Sigma Aldrich, Poole, UK)
- Non-fat dried milk (MARVEL)
- Pefabloc (Boehringer-Mannheim, Mannheim, Germany)
- PepstatinA (Sigma Aldrich, Poole, UK)
- Prestained molecular weight markers (Sigma Aldrich, Poole, UK)
- Tween-20 (Sigma Aldrich, Poole, UK)

2.3.2 Buffers

Lysis buffer: 50mM HEPES pH 7.5, 100mM NaCl, 1% Triton X100, 1mM EDTA, 1mM EGTA, 20mM NaF, 1mM Na orthovanadate, Aprotinin 10 µg/mL, Pepstatin 10 µg/mL, Leupeptin 10 µg/mL, 5µM Microcystin and 1mM Pefabloc.

Gel running buffer: Tris base 30.3g, Glycine 144.2g, SDS 10g, made up to 1L with dd H₂O.

Transfer buffer: Tris base 30.3g, Glycine 144.2g, Methanol 100mLs, made up to 1L with dd H₂O.

4x Loading buffer: 17.5 mLs 1M Tris pH 6.8, SDS 4g, DTT 2.315g, Glycerol 20 mLs, Bromophenol blue 50mg, made up to 50 mLs with dd H₂O.

2.3.3 Acrylamide gel

10% separating gel: 2.86 mls deionised water, 3.74 mLs 1M Tris pH 8.8, 3.34 mLs acrylamide/N'N'-bis-methylene 30%:0.8%, 100 µL 10% SDS, 75 µL 10% APS, 9 µL TEMED.

Stacking gel: 3.49 mls deionised water, 0.625 mls 1M Tris pH 6.8, 0.835 mLs Acrylamide/NN'methylene bis-acrylamide 30%:0.8%, 50 µL 10% SDS, 38 µL 10% APS, 7.5 µL TEMED.

Gels were prepared immediately prior to use. The separating gel was prepared as above and poured into a Mighty-Small Hoeffer gel caster (Hoeffer Scientific Instruments, San Francisco, USA). Isobutanol was layered on top of the separating gel and the solution allowed to polymerise at room temperature. The isobutanol layer was then removed, the gel was washed in ddH₂O and the stacking gel was layered on top of the separating gel and a 10 well comb inserted. Prestained molecular size markers and approximately 20 µL of sample (boiled for 5 minutes) were loaded onto the gel which was then run at 150V until adequate separation. The gel was then transferred to a Hybond-C-Extra nitrocellulose membrane by electroblotting at 0.8mA/cm² for 1 hour.

2.3.4 Antibodies

- Alpha-Tubulin (Cell Signalling Technology, Hitchin, UK)
- Mouse IgG, HRP-linked (Cell Signalling Technology, Hitchin, UK)
- Rabbit IgG, HRP-linked (Cell Signalling Technology, Hitchin, UK)
- APC conjugated mouse IgG (BD-Biosciences, Oxford, UK)
- APC conjugated CD11b (Abcam, Cambridge, UK)
- FLT3 (Cell Signalling Technology, Hitchin, UK)
- PE conjugated CD34 (BD-Biosciences, Oxford, UK)
- Phospho-Akt (Thr 308) (Cell Signalling Technology, Hitchin, UK)
- Phospho-p42/44ERK (Thr 202/Tyr204) (New England Biolabs, Hitchin, UK)
- Phospho-S6 Ribosomal Protein (Ser235/236) (Cell Signalling Technology, Hitchin, UK)

- Phospho-STAT5 (Tyr694) (Cell Signalling Technology, Hitchin, UK)

2.3.5 Flow cytometry

The methods for flow cytometry are describe in the specific materials and methods section of Chapter 7.

CHAPTER 3: SCREENING FOR MUTATIONS IN THE TYROSINE KINASE DOMAIN OF FLT3

3.1 Introduction

Protein tyrosine kinases (TKs) are an important class of enzyme that catalyse the transfer of phosphates to tyrosine residues in protein substrates, converting adenosine triphosphate (ATP) to adenosine diphosphate in the process. Tyrosine phosphorylation is an important mechanism for the regulation of protein function, thereby controlling key cellular functions such as proliferation, apoptosis and differentiation. There are over 90 different TK genes that have been identified in the human genome, with many other TK-like genes in addition to this. They can be broadly divided into two groups, receptor TKs (RTKs) at the cell surface and intracellular non-receptor TKs.

It is over 25 years since protein TKs were first implicated in oncogenesis, and subsequently the identification of aberrant protein TK signaling pathways in many different human cancers has led to important diagnostic and therapeutic advances (Krause and Van Etten 2005). A classic example is FLT3 (Fms-like tyrosine kinase 3), also known as foetal liver kinase-2 (FLK-2) or stem cell kinase-1. FLT3 is a 993 amino acid RTK encoded by a 24 exon gene located on chromosome 13 (13q12) which was independently cloned by two groups (Matthews *et al*, 1991, Rosnet *et al*, 1991). It is a class III RTK and is closely related to other members of this family of receptor TKs (Table 3.1).

Table 3.1: Members of the class III receptor tyrosine kinase family

Receptor	Alternative Names	Ligand
Fms-like tyrosine kinase 3 (FLT3)	FLK-2, stem cell kinase-1, CD135	FLT3 ligand
Steel factor receptor (c-KIT)	SCF-1, CD117	SCF
Platelet derived growth factor receptor (PDGFR) α	CD140a	PDGF-A
PDGFR β	CD140b	PDGF-A,B,C
Macrophage colony stimulating factor receptor	CSF-1R, c-FMS, CD115	CSF-1

SCF indicates stem cell factor; PDGF, platelet derived growth factor; and CSF-1R, colony stimulating factor 1 receptor

3.1.1 Structure of FLT3

The crystal structure of FLT3 has been determined (Griffith *et al*, 2004). It has all the key structural features which are associated with class III RTKs (Figure 3.1A):

- An extracellular ligand-binding domain composed of 5 immunoglobulin like domains.
- A transmembrane domain
- A juxtamembrane (JM) domain
- An activation loop
- Two TK domains or lobes linked by a kinase insert
- An ATP binding pocket within the first kinase domain.

Amino acid residues 572 to 603 encode the JM domain, whereas 604 to 946 include the activation loop, TK domains and insert. The N-lobe or first kinase domain (formed by amino acids 604-690) consists of a twisted 5-stranded β -pleated sheet adjacent to an α -helix. Within this, the ATP binding pocket is formed by residues 610-631. The C-lobe or second kinase domain (formed by residues 698-946) consists of 7 α -helices and 3 β -pleated sheets. The catalytic loop of the kinase is within this second kinase domain (residues 805-820). The 2 lobes are joined by a flexible kinase insert domain which allows considerable movement of the 2 kinase domains relative to each other. When the 2 lobes are rotated away from each other, the kinase is held in an inactive state. This inactive conformation is stabilised by an activation loop (residues 829-856 within the second kinase domain) which sits between the 2 lobes, a conformation that is typical of class 3 RTKs. Furthermore, the JM domain also acts to stabilise this conformation by making contact with virtually every structural component involved in the activation/inactivation cycle of FLT3, and is therefore critical for holding the intracellular FLT3 kinase in an inactive state (Griffith *et al*, 2004).

FLT3 is post-translationally modified and is found in two main forms, an unglycosylated 130-143 kDa non-membrane bound form and a 158-160 kDa glycosylated membrane bound form. FLT3 is expressed on the surface of normal HPCs within the BM, thymus and liver, on the surface of more mature blood cells such as monocytes, in lymph nodes and the thymus as well as in the brain, gonads and placenta. Within the BM, it is unclear whether FLT3 is expressed on the earliest HSCs but is expressed at the multipotent progenitor stage. The exact subclass of progenitor cells that

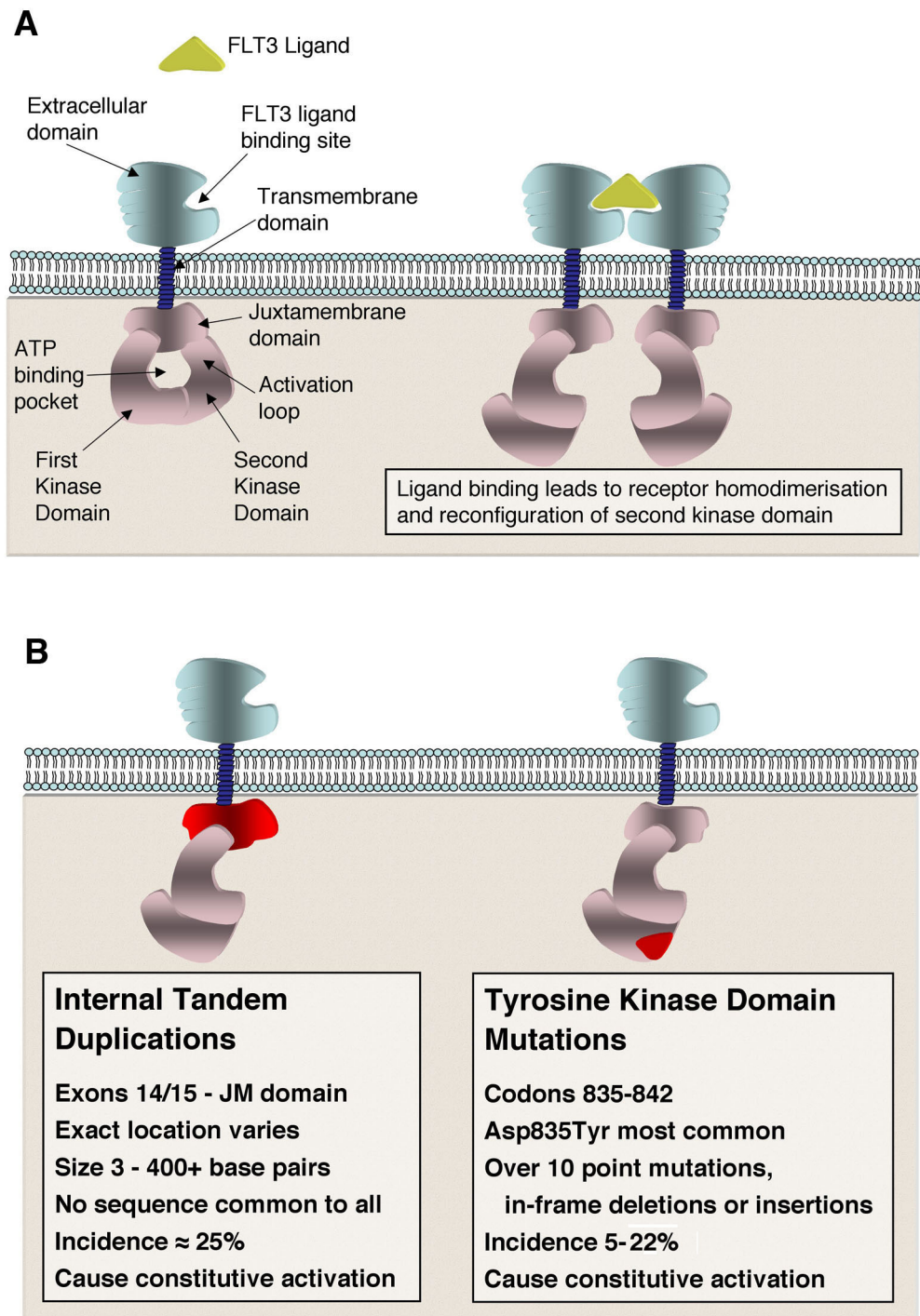


Figure 3.1. (A) Diagrammatic representation of the key structural features of the class III receptor tyrosine kinase FLT3 and (B) the key abnormalities of the two main types of mutation, internal tandem duplications and tyrosine kinase domain mutations.

express FLT3 and the lineage differentiation capability of these FLT3⁺ HPCs remains controversial in both humans and mice (Adolfsson *et al*, 2005, Luc *et al*, 2007).

3.1.2 FLT3 ligand

FLT3-ligand (FL) is a 235 amino acid type I transmembrane protein that is widely expressed as a membrane-bound and soluble form (Lyman *et al*, 1993, Stirewalt and Radich 2003). The highest expression level of FL is found in peripheral blood mononuclear cells. Ligand binding to the FLT3 protein leads to receptor homodimerisation and results in a change in the normal autoinhibitory configuration of the JM domain and activation loop. This leads to exposure of the phosphoryl acceptor sites in the kinase domains and consequent autophosphorylation of a number of key regulatory intracellular tyrosine residues in FLT3. These changes lead to a reorientation of critical amino acids, thereby increasing the catalytic activity of the enzyme and generating binding sites for protein substrates. The activated receptor is rapidly internalised and degraded.

3.1.3 Mutations in the JM domain of FLT3

Interest in FLT3 in AML was initially generated by the demonstration of expression of the receptor on the surface of blast cells from the majority of cases of AML (Drexler 1996, Rosnet *et al*, 1996). It is also expressed in acute lymphoblastic leukaemia (ALL) and blast crisis of chronic myeloid leukaemia (CML). The crucial role for FLT3 in AML leukaemogenesis became apparent when a study screening AML samples for expression of FLT3 using reverse-transcriptase polymerase chain reaction (PCR) noted that a number of samples had unexpectedly long transcripts in the JM domain (Nakao *et al*, 1996). It was subsequently shown that these long transcripts were due to a region of duplicated genomic DNA within the JM domain, excluding aberrant splicing as an explanation for this finding. The duplications were always in-frame and located in the JM domain, but varied considerably in length and exact location from patient to patient. The mutations are somatic mutations as demonstrated by the absence of the FLT3/ITD in remission samples from patients who were FLT3/ITD positive at diagnosis. Screening of larger cohorts of patients demonstrated that these internal tandem duplications (ITDs) of FLT3 occur in approximately one quarter of cases of AML in young adults (Kottaridis *et al*, 2003, Levis and Small 2003, Stirewalt and Radich 2003). Interestingly, 9-23% of FLT3/ITD mutant positive patients have been reported to carry more than one duplication, and up to 5 have been reported in a single patient (Kottaridis

et al, 2003). The presence of FLT3/ITDs is generally restricted to cases of AML, although occasional cases in myelodysplasia, chronic myelomonocytic leukaemia and ALL have been reported (Lee *et al*, 2007, Xu *et al*, 1999, Yokota *et al*, 1997).

As shown in Figure 3.1B, the ITDs interfere with the normal autoinhibitory role of the JM domain (Griffith *et al*, 2004), and functional studies have shown that the presence of a FLT3/ITD leads to constitutive activation of the receptor (Stirewalt and Radich 2003). The clinical and functional consequences of FLT3/ITDs in AML are discussed in more detail in chapters 4, 5 and 7 respectively. Interestingly, two studies have also identified point mutations within the JM domain in primary AML samples and myeloid cell lines (Reindl *et al*, 2006, Stirewalt *et al*, 2004) that were shown to lead to constitutive activation of FLT3 in a murine cell line model (Reindl *et al*, 2006). These types of mutation are, however, uncommon in AML, occurring in <1% of the patients studied.

3.1.4 Mutations in the TK domains of FLT3

Subsequent to the identification of FLT3/ITDs in AML, it was noted that primary AML blasts from a number of patients had constitutively phosphorylated FLT3 in the absence of a FLT3/ITD (Fenski *et al*, 2000) raising the possibility of other mechanisms of aberrant FLT3 activation. Shortly afterwards, two groups independently reported activating mutations affecting codons D835 and I836 in the second TK domain (TKD) of FLT3 in approximately 7% of AML patients (Abu-Duhier *et al*, 2001, Yamamoto *et al*, 2001). These FLT3/TKD mutations also occur at a lower frequency in ALL and myelodysplasia (Yamamoto *et al*, 2001). This part of the molecule was screened as mutations have been reported to occur in the highly conserved homologous region of the closely related receptors c-KIT and FMS. For example, D816 mutations of c-KIT had been reported to occur in AML, mast cell lines and systemic mastocytosis (Furitsu *et al*, 1993, Kitayama *et al*, 1995, Longley *et al*, 1999, Sotlar *et al*, 2000, Sperr *et al*, 1998). Mutations of FMS at codon D802 had also previously been shown to be constitutively activating (Figure 3.1B) (Sotlar *et al*, 2000). Interestingly, one group had already shown that substitution of aspartate with valine at position 838 of the murine FLT3 kinase (the equivalent to human FLT3 codon 835) led to constitutive activation (Fenski *et al*, 2000). Similar to FLT3/ITDs, the TKD mutations of FLT3 were shown to be somatic mutations as they were absent in remission samples. Furthermore, screening of normal controls did not detect any TKD mutations. It is thought that TKD mutations

disrupt key amino acids which stabilise the activation loop in a closed configuration, although the exact mechanism of this disruption remains unclear (Griffith *et al*, 2004).

The presence of FLT3/TKD mutations within the second TK domain in AML has been confirmed in a number of other studies (Table 3.2). The overall incidence in AML has been reported to be approximately 7%, ranging between 5% and 22% depending on the technique used and the specific inclusion criteria of the study with regards to patient age, cytomorphology and karyotype. A number of different mutations have been reported. Most are point mutations such as substitution of aspartate residue 835 with a tyrosine (D835Y, the most frequent mutation), histidine, valine or glutamate; other alterations include small deletions (Δ 835, Δ 836) and insertions. All mutations initially described disrupted an *EcoRV* restriction enzyme cutting site that facilitated a simple screening technique using digestion of PCR products, an approach which has been employed in most studies (Table 3.2). Subsequently, however, using different techniques to screen the whole of exon 20 of FLT3, a number of other point mutations have been reported at codons 839, 841 and 842 (Jiang *et al*, 2004, Kindler *et al*, 2005, Smith *et al*, 2005) as well as a two amino acid insertion between codons 840 and 841 (Spiekermann *et al*, 2002). All of these mutations occur outside of the *EcoRV* restriction digest site and would therefore have been missed using this technique. Interestingly, a single case of a constitutively activating point mutation in the first TK domain has also been described (K663Q) (Schittenhelm *et al*, 2006). Further studies will be required in order to determine the frequency of such mutations.

3.1.5 Screening techniques for the detection of FLT3/TKD mutations

In order to study the clinical consequences of these relatively infrequent FLT3/TKD mutations, screening of large cohorts of patients with AML is required. A number of different techniques have been used for the detection of FLT3/TKD mutations and each has specific limitations. For example, direct sequencing lacks sensitivity for the detection of somatic mutations which may only be present in a subclone of cells. Techniques depending on the *EcoRV* restriction digest are limited by the failure to detect mutations occurring within the TKD but outside of the digest site. Other techniques such as SSCP and CSGE are technically demanding and therefore impractical for large scale screening programmes. Lightcycler melting curve analysis depends on the exact sequence of the sensor and anchor probes used, for example, in the

Table 3.2: Published studies reporting TKD mutations of FLT3 in AML

Reference	No. patients	Method	No. TKD Mutant (%)	Mutation, No.	Multiple Mutants, No.
(Abu-Duhier <i>et al</i> , 2001)	97	Sequencing, <i>EcoRV</i> , and CSGE	7 (7.2)	D835Y, 5 Δ835, 1 D835H, 1	None
(Yamamoto <i>et al</i> , 2001)	429	<i>EcoRV</i> and sequencing	30 (7.0)	D835Y, 23 D835V, 5 D835E, 2 D835H, 1	D835E + D835Y, 1
(Thiede <i>et al</i> , 2002)	979	<i>EcoRV</i> and sequencing	75 (7.7)	D835Y, 34 D835H, 18 D835V, 5 D835E, 8 D835N, 2 D835G, 4 D835LK, 1 Δ836, 13 I836MR, 1 I836T, 1	dual mutants, 8 triple mutants, 1
(Noguera <i>et al</i> , 2002)	90 (APL only)	<i>EcoRV</i> and sequencing	7 (7.8)	D835Y, 3 D835E, 1 NR, 3	None
(Frohling <i>et al</i> , 2002)	224	<i>EcoRV</i> and sequencing	32 (14.3)	D835Y, 17 D835E, 6 D835V, 2 D835H, 2 D835A, 1 Δ836, 2 NR, 1	1, D835Y + Δ836
(Spiekermann <i>et al</i> , 2002)	359	PCR and sequencing	N/A	Ins840GS, 2	N/A
(Sheikhha <i>et al</i> , 2003)	80	<i>EcoRV</i> and Sequencing	6 (7.5)	D835Y, 4 D835E, 2	None
(Bianchini <i>et al</i> , 2003)	34	<i>EcoRV</i> , Sequencing and dHPLC	2 (5.9)	D835E, 2	None
(Shih <i>et al</i> , 2003)	107 (APL only)	<i>EcoRV</i> and sequencing	20 (18.7)	D835Y, 13 D835H, 5 D835V, 1 D835E, 1	None
(Moreno <i>et al</i> , 2003)	208	<i>EcoRV</i> and sequencing	20 (9.6)	D835Y, 5 D835H, 3 D835V, 1 D835A, 1 NR, 10	None
(Jiang <i>et al</i> , 2004)	53	Sequencing, mass spectrometry	NR	N841I, 2 N841Y, 1	1, N841I and D835 point mutation
(Andersson <i>et al</i> , 2004)	109	<i>EcoRV</i> and sequencing	11 (10.1)	D835Y, 3 D835V, 2 D835H, 2 D835N, 1 D835E, 1 Δ834 and 835, 1 NR, 1	None
(Au <i>et al</i> , 2004)	82 (APL only)	<i>EcoRV</i> and sequencing	18 (22.0)	D835Y, 11 D835V, 4 D835H, 3 D835A, 1	None

Table 3.2: continued

Reference	No. patients	Method	No. TKD Mutant (%)	Mutation, No.	Multiple Mutants, No.
(Shih <i>et al</i> , 2004)	120	<i>EcoRV</i> and sequencing	13 (10.8)	D835Y, 9 D835V, 2 D835E, 1 D835H, 1	None
(Mills <i>et al</i> , 2005)	258	Capillary electrophoresis and sequencing	35 (13.6)	D835/836 Pt M, 30 Δ836, 2 D839, 2	1, Triple mutant
(Scholl <i>et al</i> , 2005)	122	<i>EcoRV</i> , Sequencing and real-time PCR	9 (7.4)	D835Y, 5 D835H, 3 Δ836, 1	None
(Smith <i>et al</i> , 2005)	175	SSCP, <i>EcoRV</i> and sequencing	8 (4.6)	D835 Pt M, 7 D839G, 1	NR
(Kindler <i>et al</i> , 2005)	110	Sequencing	9 (8.2)	D835 Pt M, 7 Y842C, 2	None
(Wang <i>et al</i> , 2005)	143	<i>EcoRV</i>	9 (6.3)	NR	NR
(Auewarakul <i>et al</i> , 2005)	256	<i>EcoRV</i> and sequencing	15 (5.9)	D835Y, 7 D835H, 4 D835E, 1 D835A, 1 Δ835/I836V, 1	None
(Mills <i>et al</i> , 2006)	N/A	Capillary electrophoresis and sequencing	N/A	D835V/I836F/M837P	N/A
(Schittenhelm <i>et al</i> , 2006)	109	dHPLC and sequencing	5 (4.6)	D835Y, 2 D835H, 1 Δ836, 1 N841H	None
(Whitman <i>et al</i> , 2008)	217	<i>EcoRV</i> and sequencing	19 (8.8)	NR	NR
(Bacher <i>et al</i> , 2008)	3082	Lightcycler melting curve analysis	147 (4.8)	D835Y, 68 D835H, 20 D835V, 14 D835E, 11 D835A, 3 D835S, 1 D835N, 1 Δ836, 14 NR, 10	D835A + D835Y, 1 D835E + D835Y, 2 D835Y + D835H, 1 D835Y + Δ836

NR, not reported; CSGE, Conformation sensitive gel electrophoresis; SSCP, Single strand conformational polymorphism; dHPLC, denaturing high performance liquid chromatography; APL, acute promyelocytic leukaemia; and Pt M, point mutation.

study by Bacher *et al* (2007) the probes used would pick up mutations within codons 821 to 838 but would fail to detect mutations occurring between codons 839 to 842.

The experimental work presented in this chapter evaluated the role of denaturing high performance liquid chromatography (dHPLC) for the detection of FLT3/TKD mutations. The Transgenomic WAVE® System of dHPLC is a powerful technique for

the detection of mutations based on temperature modulated heteroduplex analysis. It was developed in the early 1990s (Xiao and Oefner 2001), and the use of dHPLC for the detection of somatic mutations was first reported for the detection of phosphatase and tensin homolog (PTEN) mutations in malignant gliomas (Liu *et al*, 1998). The technique involves binding a PCR product to a polystyrene-divinylbenzene copolymer column using tri-ethylene ammonium acetate (TEAA) an ion pairing agent. The column contains hydrophobic beads that interact with the hydrophobic portion of TEAA, which in turn binds to negatively charged DNA via its positively charged phosphate backbone. The temperature of the column is carefully calculated in order to partially denature the double stranded DNA. Gradually increasing concentrations of acetonitrile elute the DNA off the column, an ultraviolet detector measures the DNA absorbance of the eluate and a chromatogram is generated. The presence of heteroduplexes in the PCR product leads to a higher proportion of the fragment being single stranded which is less strongly bound and therefore elutes off the column earlier. The presence of heteroduplexes formed by the presence of a somatic mutation will therefore be detected by an abnormal chromatogram pattern (Figure 3.2). A major advantage of this technique is that it is automated and relatively rapid (approximately 7 minutes per sample). The accuracy of dHPLC for the detection of FLT3/TKD mutations was therefore first evaluated and compared with the most commonly used alternative screening technique, *EcoRV* restriction digest analysis, and then applied to a large cohort of samples from adult patients with AML.

3.2 Patients, Materials and Methods

3.2.1 Patients

Genomic DNA (gDNA, n=1297) or complementary DNA (cDNA, n = 42) was available from blast cells of 1339 adults with AML entered into either the UK MRC AML 10 (n=444) or AML12 (n=895) trials (further details on the clinical characteristics and treatment of these patients in chapter 4). Ethical approval for the trials and tissue collection for research was obtained from the Multi-Centre Research Ethics Committee of Wales and local research ethics committees as appropriate and informed consent was provided according to the Declaration of Helsinki.

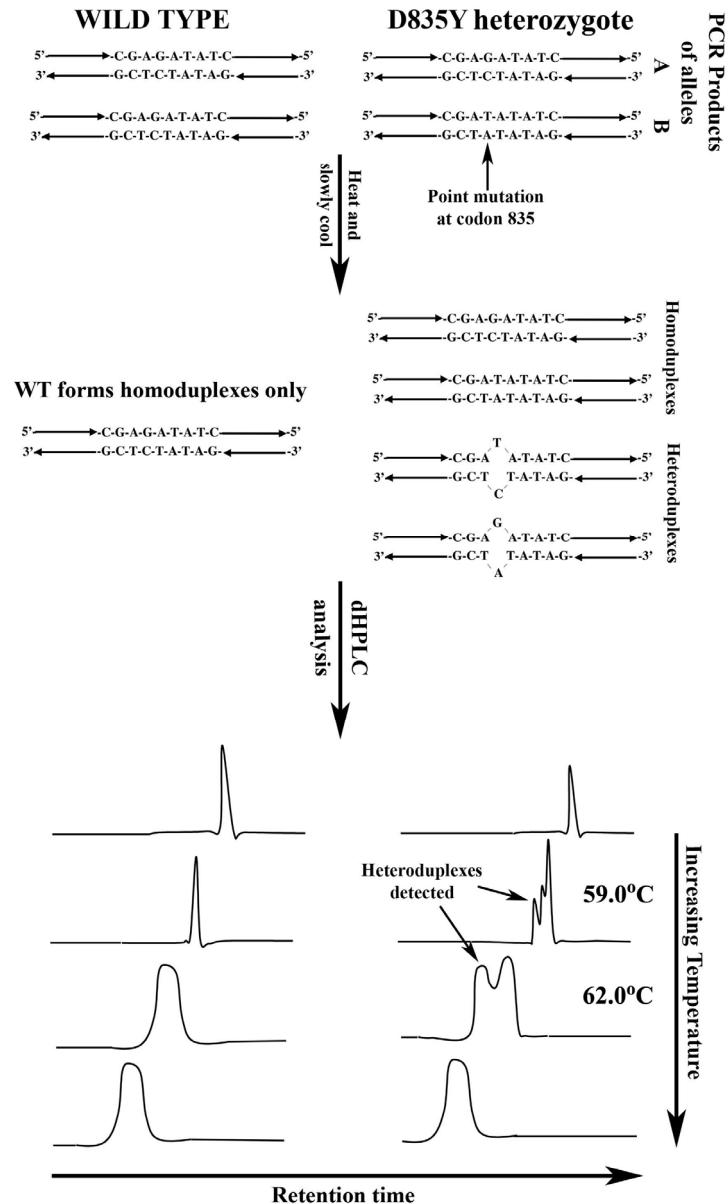


Figure 3.2. Schematic representation of FLT3/TKD mutation detection by dHPLC. PCR amplification of wild type and heterozygous D835Y mutant (allele A is FLT3/WT, B is D835Y mutant) cases generates double stranded DNA fragments containing the respective mutant and wild-type alleles. These products are then heated and slowly cooled, generating heteroduplexes in the mutant case only. The lower section shows a diagram of dHPLC. At too low a temperature, all the fragments are double stranded and have a long retention time (time taken to elute off the column). As the temperature increases, heteroduplexes are resolved as the PCR products are partially denatured. At too high a temperature, all the fragments are single stranded and the retention time is therefore short.

3.2.2 Screening for FLT3/TKD mutations by dHPLC

For dHPLC analysis to screen for FLT3/TKD mutations, PCR was used to amplify a 278 base pair (bp) fragment covering FLT3 exon 20 (previously designated exon 17) and the flanking intronic regions from approximately 100ng gDNA with Optimase polymerase using manufacturer's recommended conditions, primers 20F and 20R (Table 3.3), 32 cycles of amplification and an annealing temperature of 63°C. For cDNA, a 290bp fragment covering exon 20 was amplified as above with primers FLT3/F2 and FLT3/R (Table 3.3). Products were denatured and run on dHPLC (Transgenomic WAVE) at optimal melting temperatures calculated using WAVEMAKER software: 59.0°C and 62.0°C for the 278bp fragment from gDNA (Figure 3.3A), 60.2°C and 62.0°C for the 290bp product from cDNA (Figure 3.3B). The higher temperature was required in order to reduce the possibility that a mutation could be missed in the section of the PCR product that remains double stranded at the lower temperature (see Figure 3.3).

In order to determine the sensitivity of dHPLC for the detection of FLT3/TKD mutations, dilutions of a D835Y mutant positive case were made with equal amounts of DNA (as measured by optical density) from the promyelocytic leukaemia cell line NB4, which is FLT3/WT. Dilutions of D835Y:NB4 of 1:1, 1:3, 3:17, 1:9 thereby should lead to an estimated percentage of D835Y mutant alleles of 25%, 12.5%, 7.5% and 5% respectively. These estimations assume a 50% level of mutant in the D835Y sample and that optical density accurately correlates with the number of FLT3 alleles available for PCR amplification in each sample.

3.2.3 Screening for FLT3/TKD mutations by *EcoRV* restriction digest

In order to partially overcome the difficulty in discriminating between a failed or partial *EcoRV* digest and a codon 835/836 FLT3/TKD mutation, a modified method of the original PCR *EcoRV* digestion procedure was used (Gale *et al*, 2005b, Yamamoto *et al*, 2001). Using the original method, *EcoRV* cuts WT sequence once but does not cut alleles with mutations of codons 835 or 836 (Yamamoto *et al*, 2001). In the modified method, a mismatch was introduced into the reverse primer (Table 3.3), thereby creating

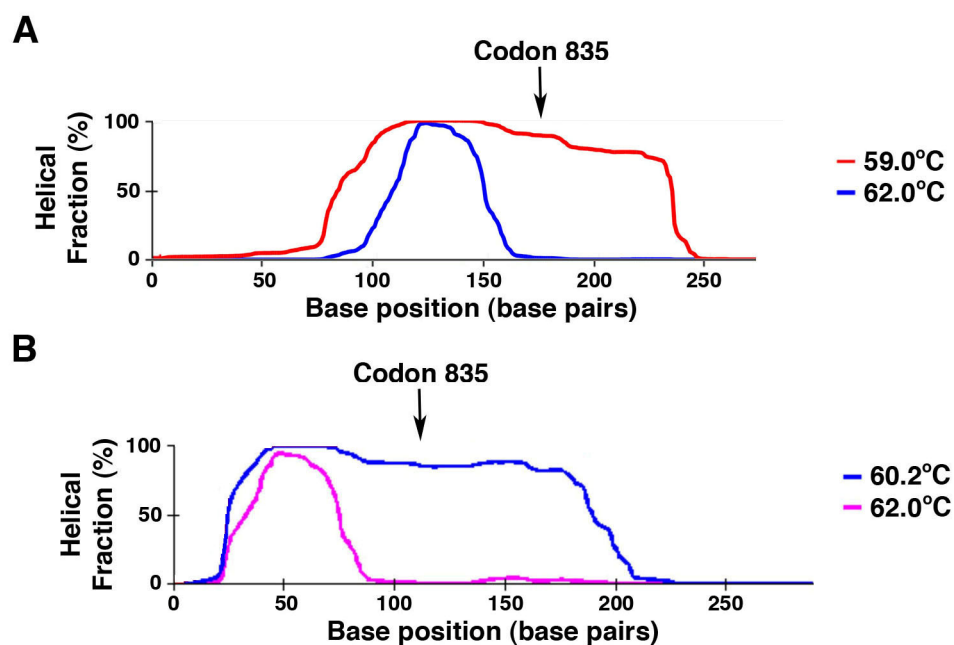


Figure 3.3. Theoretical melting curves of PCR products generated by WAVEMAKER software. (A) 278 bp PCR product generated from genomic DNA run at 59.0°C and 62.0°C. Exon 20 includes base pairs 100 to 223 of this PCR fragment. The location of codon 835 is shown. (B) 290 bp PCR product generated from cDNA run at 60.2°C and 62.0°C. Mutation detection is optimal when the helical fraction of the area where the mutations reside is between 75 and 95%.

Table 3.3: PCR primers and restriction enzyme digests used for the detection of FLT3/TKD mutations

	Primers	PCR Annealing Temperature	RE	WT PCR product size	MT fragment lengths post digest
DHPLC					
gDNA	20F: 5'-CATCACCGGTACCTCCTACTG-3' 20R: 5'-TAACGACACAACACAAAATAGCCGT-3'	63°C	N/A	278bp	N/A
cDNA	FLT3/F2: 5'-GGAATGGAATTTCTGGAATTTAAGTCG-3' FLT3/R: 5'-ACCGGAATGCCAGGGTAAGGA-3'	63°C	N/A	290bp	N/A
EcoRV					
gDNA	20F: 5'-CATCACCGGTACCTCCTACTG-3'	63°C	<i>EcoRV</i>	303bp	26 + 277
	20R2 (mm): 5-CAGTGAGTGCAGTTGTTTACCATGATATCG-3				
Mutation					
D835Y	F(mm): 5'-GTGAAGATATGTGACTTTGGATTGGATCGA-3' R: 5'-CAGTGAGTGCAGTTGTTTACCATGATAACG-3'	62°C	<i>ClaI</i>	149bp	27bp + 122bp
D835H	F(mm): 5'-GTGAAGATATGTGACTTTGGATTGGGTCGA-3' R: 5'-CAGTGAGTGCAGTTGTTTACCATGATAACG-3'	64°C	<i>HincII</i>	149bp	28bp + 121bp
D835E	F: 5'-CATCACCGGTACCTCCTACTG-3' R: 5'-TAACGACACAACACAAAATAGCCGT-3'	63°C	<i>DpnII</i>	278bp	188bp + 90bp
D835V	F: 5'-CATCACCGGTACCTCCTACTG-3' R(mm): 5'-CCTGACAACATAGTTGGAATCACTCATGTT-3'	64°C	<i>HincII</i>	217bp	187bp + 30bp
Δ836	F(mm): 5'-AAGATATGTGACTTTGGATTGGCTCGACAT-3' R: 5'-CAGTGAGTGCAGTTGTTTACCATGATAACG-3'	64°C	<i>NdeI</i>	146bp	29bp + 114bp
Δ835	F(mm): 5'-GTGAAGATATGTGACTTTGGATTGGTTTCGA-3' R: 5'-CAGTGAGTGCAGTTGTTTACCATGATAACG-3'	64°C	<i>BstBI</i>	149bp	27bp + 119bp
D835N	F(mm): 5'-GTGAAGATATGTGACTTTGGATTGGTTTCGA-3' R: 5'-CAGTGAGTGCAGTTGTTTACCATGATAACG-3'	64°C	<i>BstBI</i>	149bp	27bp + 122bp

F indicates forward primer, R reverse primer, WT wild type, MT mutant, mm mismatch primer (mismatch underlined), RE restriction endonuclease, N/A not applicable.

an additional *EcoRV* digestion site that would be present in all PCR products, irrespective of the presence of the mutation. This allowed discrimination of undigested products from FLT3/TKD⁺ cases (cut once) and FLT3/WT cases (cut twice). For gDNA, 35 cycles of amplification were performed with an annealing temperature of 63°C. *EcoRV* digestion of the 180 bp product gave bands of 277 and 26 bp for FLT3/TKD mutant alleles and 187 + 90 + 26 bp for WT alleles. Mutation screening on cDNA samples using *EcoRV* digestion was carried out in previous work in the department at UCL (Gale *et al*, 2005b). In total, 430 patients were screened for FLT3/TKD mutations by *EcoRV* restriction digest, including all patients with an abnormal WAVE chromatogram.

3.2.4 Identification of TKD Mutations

PCR products were obtained using 35 cycles of amplification with BIOTAQ DNA polymerase (Bioline, London, UK), manufacturer's recommended conditions and annealing temperatures as specified (Table 3.3). They were sequenced using the DTCS Quick Start kit and analysed on a CEQ8000 DNA Genetic Analysis System (Beckman Coulter). Some mutations were confirmed using mutation-specific restriction enzyme digestion of PCR products by designing mismatch primers that discriminated between WT and mutant alleles (Table 3.3). For samples where the mutant level was too low to be identified by sequencing or restriction digest, PCR products were cloned (TOPO TA Cloning, Invitrogen) and sequenced.

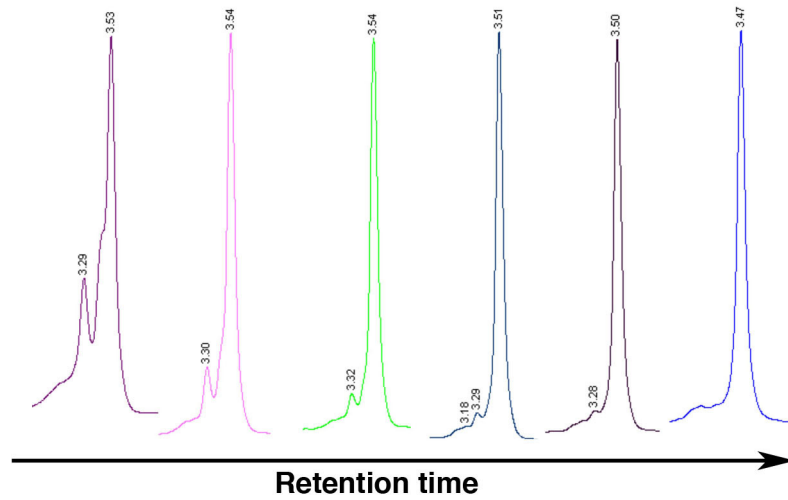
3.3 Results

3.3.1 Sensitivity of dHPLC for the detection of D835Y mutations

In order to determine the sensitivity of dHPLC for the detection of FLT3/TKD mutants, mixtures of gDNA were made from the FLT3/WT cell line NB4 and a known D835Y mutant case where approximately half the FLT3 alleles were known to carry a mutation. At a melting temperature of 59.0°C, mutations could be detected down to a 9:1 ratio of NB4:D835Y heterozygous mutant, consistent with approximately 5% of the FLT3 alleles carrying a mutation (Figure 3.4A). The higher melting temperature of 62.0°C was less sensitive for the detection of the D835Y mutation (Figure 3.4B) as the relevant section of the PCR fragment is completely single stranded at this temperature (Figure 3.3A).

A

Mutant:NB4	100:0	50:50	25:75	15:85	10:90	0:100
%Mutant	50%	25%	12.5%	7.5%	5%	0%

**B**

Mutant:NB4	100:0	50:50	25:75	15:85	0:100
%Mutant	50%	25%	12.5%	7.5%	0%

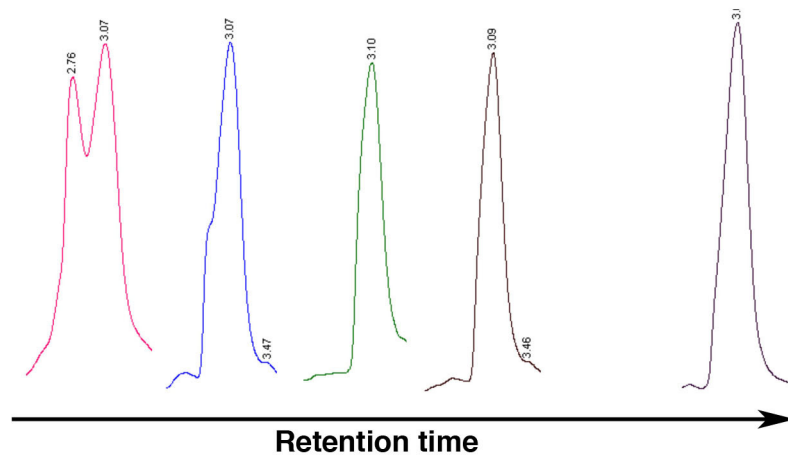


Figure 3.4. dHPLC chromatogram traces of PCR products amplified from mixtures of genomic DNA from a heterozygous D835Y mutant positive case and from the FLT3 wild-type NB4 cell line. (A) 59.0°C and (B) 62.0°C.

3.3.2 Incidence of FLT3/TKD mutations

In the total cohort of 1339 patients studied, 161 (12%) had an abnormal chromatogram trace, indicative of the presence of a FLT3/TKD mutation (FLT3/TKD⁺). As predicted from Figure 3.4, when using gDNA the chromatogram trace at 59.0°C was the most sensitive for mutation detection and the most robust for the specific patterns associated with specific mutants (Figure 3.5A - O). The higher temperature (62.0°C) was useful for confirmation of an abnormal trace at the lower temperature. It is also possible that, given the melting temperature profiles shown in Figure 3.3, mutations in the region of the amplified fragment that remains tightly double stranded at 59.0°C would be missed at this temperature. Therefore all samples were run at both temperatures. The chromatogram profiles of the 42 cases screened from cDNA samples were more difficult to optimise but, nevertheless, picked up all 4 mutants previously detected in these cases using *EcoRV* screening in prior work in the department (Figure 3.5 R, S) (Gale *et al*, 2005b).

3.3.3 Identification of FLT3/TKD mutations

Mutations were initially determined by direct sequencing, however, it soon became apparent that the WAVE chromatogram pattern was often specific to the underlying mutation. In many cases therefore the mutation could be confirmed by mutation-specific restriction enzyme digest without the need for sequencing (Tables 3.3 and 3.4, Figure 3.5B, C, D, E, F, I, J). All mutants were identified and confirmed except those in 8 patients in which the heteroduplex peak was very small, suggesting that the mutant present was only a small percentage of the total FLT3 alleles. In four of these patients, *EcoRV* restriction digest screening was positive. In the other four cases the *EcoRV* digest was negative but the chromatogram traces were consistently and reproducibly abnormal and these cases were therefore scored as mutant-positive.

Overall, 14 different mutations were identified (Table 3.4, Figure 3.5). D835Y was the most common, accounting for 50% of mutant-positive patients; D835H, D835V, D835E and Δ836 were all found at a similar frequency of 8%-11% of positive patients. Three previously undescribed mutations were detected, each in a single case: S840G (Figure 3.5L), a 3 bp insertion which replaced aspartate codon 835 with glycine and proline (Figure 3.5G), and a 16bp tandem duplication plus 10bp deletion within the kinase domain which replaced codons 835 and 836 (DI) with VIPT (Figure 3.5H).

Table 3.4: FLT3/TKD mutations detected in total cohort of 1339 patients.

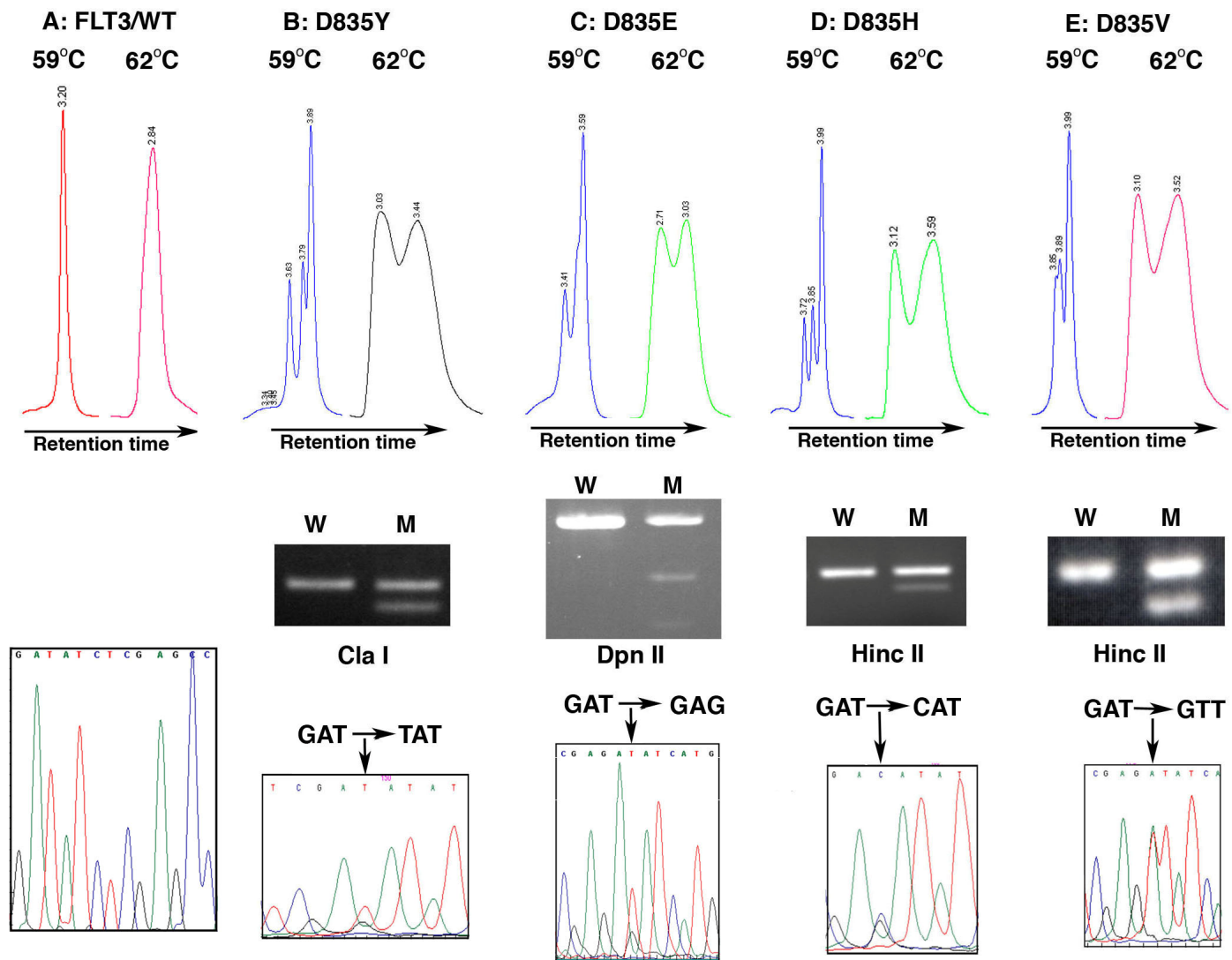
Where 2 mutations were present, the case is classified according to the predominant mutation.

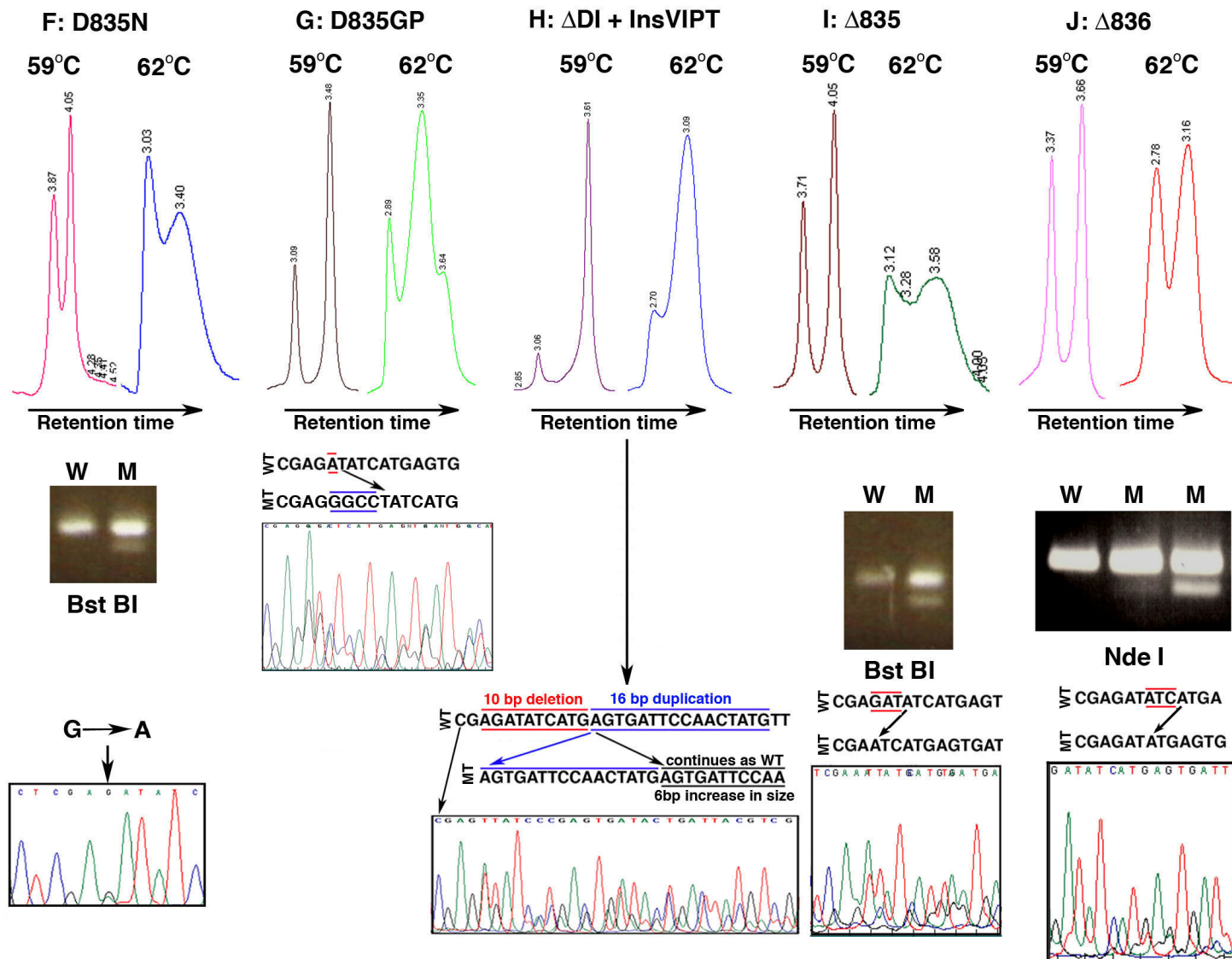
Mutation	Total (%FLT3/TKD⁺ Patients)	WAVE Pattern
D835Y	50 (50%)	Figure 3.5B
D835E	13 (8%)	Figure 3.5C
D835H	18 (11%)	Figure 3.5D
D835V	15 (9%)	Figure 3.5E
D835N	1 (1%)	Figure 3.5F
D835GP	1 (1%)	Figure 3.5G
ΔDI + InsVIPT	1 (1%)	Figure 3.5H
Δ835	1 (1%)	Figure 3.5I
Δ836	16 (10%)	Figure 3.5J
D839G	1 (1%)	Figure 3.5K
S840G	1 (1%)	Figure 3.5L
N841I	2 (1%)	Figure 3.5M
N841K	2 (1%)	Figure 3.5N
N841Y	1 (1%)	Figure 3.5O
Unknown	8 (5%)	Figure 3.6

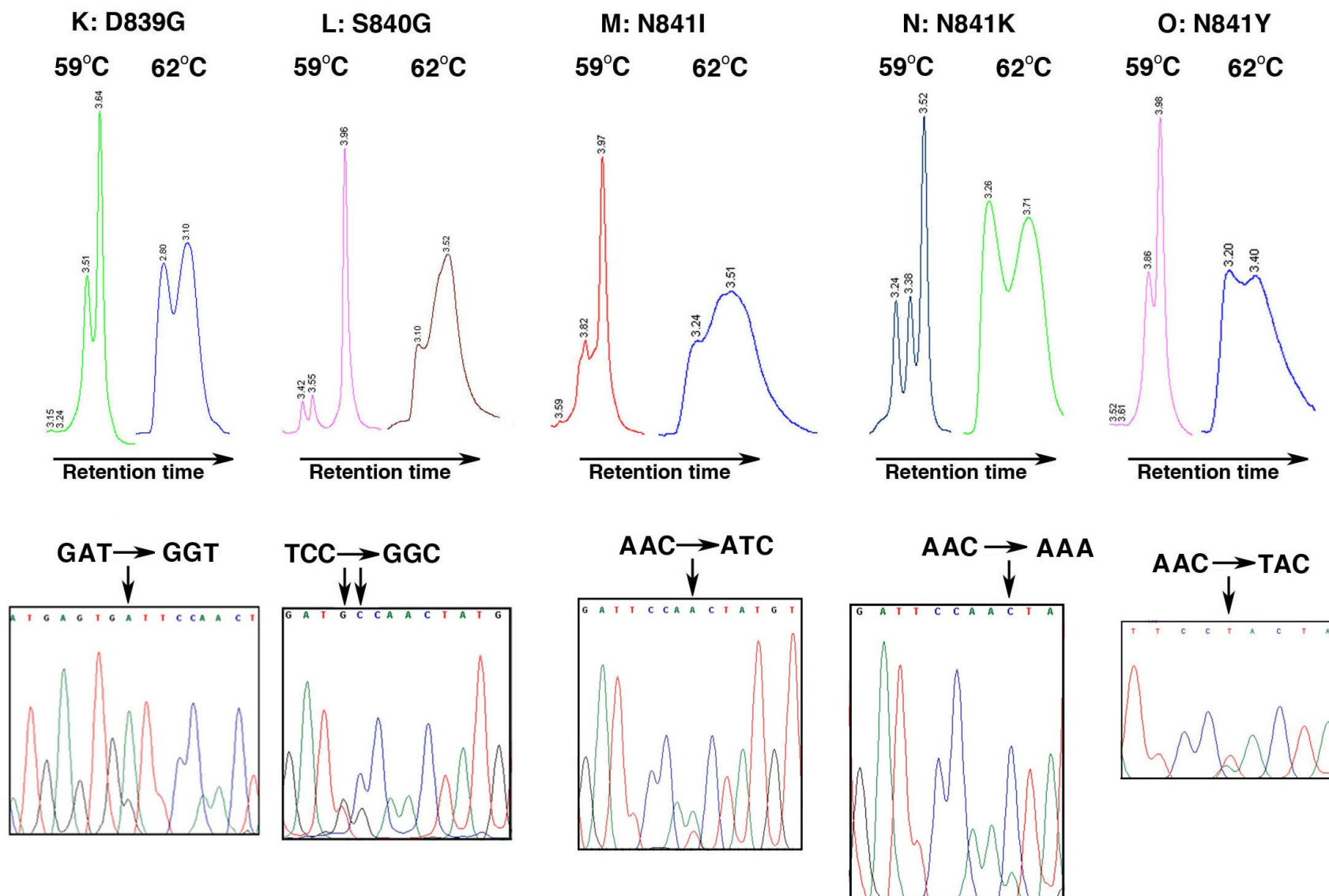
Of the 161 cases with an abnormal chromatogram, 155 had a pattern consistent with a single mutant and six samples had a pattern suggestive of the presence of two mutants (Figure 3.5P). In each of these six cases, there was a predominant mutant which was readily identified and a minor mutation that was identified in 4 of the cases. The mutation types (predominant followed by the minor mutant) were D835Y and Δ836 (shown in Figure 3.5P); D835H and D835Y; Δ836 and D835H; N841K and D835Y; N841I and unknown (*EcoRV* positive); D835V and unknown (*EcoRV* negative).

A technical issue relating to sample quality and dHPLC analysis is demonstrated in Figure 3.5Q. Some of the aliquots of gDNA in the UCL DNA bank are more than 10 years old. The working DNA solutions were stored as dilutions of a main stock of gDNA. In a number of cases the working DNA had degraded and this created an abnormal dHPLC chromatogram, however, no mutation could be detected by sequencing. In these cases, when a fresh aliquot of the main stock gDNA was made, the dHPLC chromatogram was normal.

Figure 3.5 (next 4 pages). dHPLC chromatogram traces of different FLT3 TKD mutations and their confirmation by specific restriction digest and/or direct sequencing. For the specific restriction digests, M represents mutant positive cases and W wild type. (A) FLT3 wild type; (B) D835Y with *Cla*I mismatch digest and relevant section of sequence; (C) D835E with *Dpn*II digest and relevant section of sequence; (D) D835H with *Hinc*II mismatch digest and sequence; (E) D835V with *Hinc*II mismatch digest and sequence; (F) D835N with *Bst*BI mismatch digest and sequence; (G) D835GP with sequence; (H) Δ DI + InsVIPT with sequence; (I) Δ 835 with *Bst*BI mismatch digest and sequence; (J) Δ 836 with *Nde*I mismatch digest and sequence; (K) D839G with sequence; (L) S840G with sequence; (M) N841I with sequence; (N) N841K with sequence; (O) N841Y with sequence; (P) dual mutation with a predominant D835Y mutation and a minor Δ 836 mutation; (Q) chromatogram trace at 59°C from a sample with degraded genomic DNA showing the corresponding agarose gel electrophoresis of the 278bp product, with the degraded case shown by the black arrow; the chromatogram trace obtained using a fresh aliquot of DNA is normal; (R) D835V mutation detected by dHPLC of PCR amplified product from cDNA; (S) D835Y mutation detected by dHPLC of PCR amplified product from cDNA.

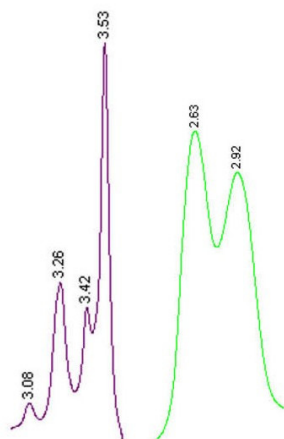






P: D835Y and Δ 836

59°C 62°C

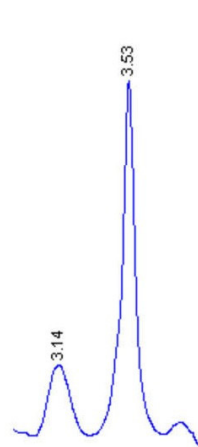


Retention time →

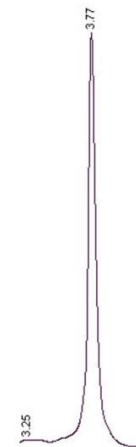
Q: Degraded DNA - FLT3/WT

Old aliquot

New aliquot



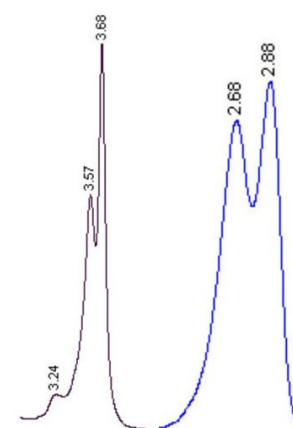
Retention time →



Retention time →

R: D835V cDNA

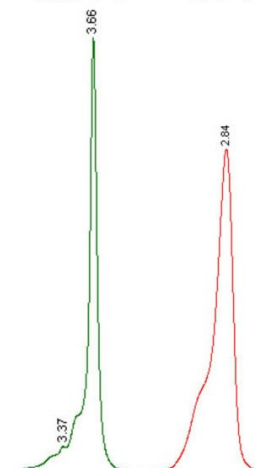
60.2°C 62°C



Retention time →

S: D835Y cDNA

60.2°C 62°C



Retention time →



3.3.4 Comparison of *EcoRV* Screening and dHPLC screening for FLT3/TKD mutations

EcoRV digestion and dHPLC analysis were performed in 430 patients, including all 161 with an abnormal WAVE pattern: 269 were WT by both techniques but 17 (11%) that were mutant by dHPLC were WT by *EcoRV* digestion (Figure 3.6). In total, seven of the 161 mutations detected (4%) occurred outside the *EcoRV* restriction digest site. Five of these cases had a markedly abnormal dHPLC chromatogram but were negative by *EcoRV*. Two further mutations that occurred outside the digest site were *EcoRV* positive due to the presence of a second minor mutation within codons 835 and 836 (see section 3.3.3). Eight cases had an identified mutation within the digest site but were negative by *EcoRV* screening (Figure 3.6). In these cases, the dHPLC trace was suggestive of a minor mutation that was below the level of detection for restriction digest analysis. A further four cases were scored as mutant-positive as their dHPLC chromatogram trace was reproducibly abnormal but they were negative by *EcoRV* screening and the mutation was not identified. Consequently, approximately one-third of the mutants missed by *EcoRV* digestion were because the mutation did not change this cutting site and the remainder were below the detection level of the *EcoRV* technique.

There were two mutant-positive cases that showed only a very slight degree of digestion by *EcoRV*, suggesting that only a small minority of the FLT3 alleles were WT. The chromatogram profile on these patients was abnormal in both cases (one example, a D835V mutation, is shown in Figure 3.6D) but only due to the presence of a ‘shoulder’ on the main peak. These cases with apparent loss of the WT allele are discussed in more detail in chapter 5.

3.4 Discussion

With an ever-increasing number of different molecular abnormalities now being described in human malignancies, developing appropriate molecular biology techniques to detect them is a major challenge. Even in AML alone, there are many different genetic mutations that have been reported (see chapter 1), and it is possible that information derived from the detection of some of these mutations will directly influence clinical management. In the case of FLT3 mutations, the introduction of FLT3 inhibitors into large clinical trials (Knapper 2007) has necessitated that the FLT3

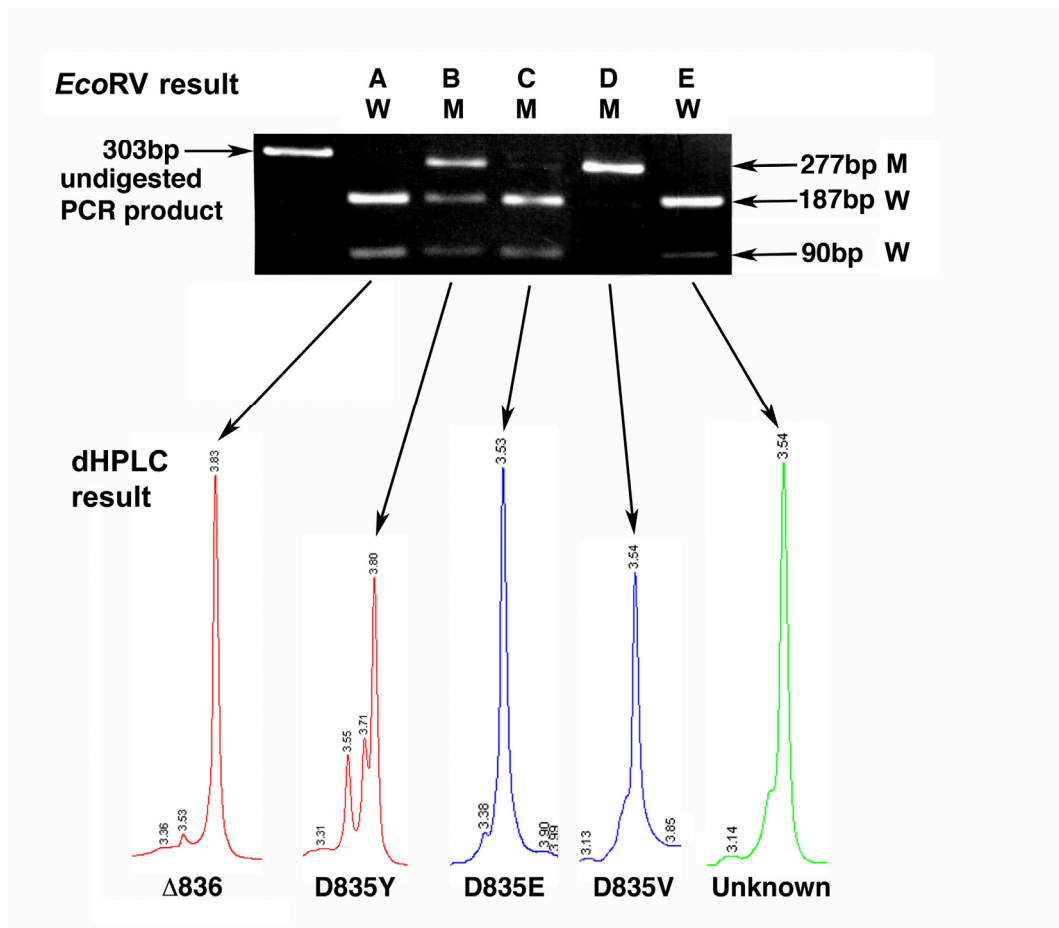


Figure 3.6. *EcoRV* restriction digest screening for FLT3/TKD mutations and the corresponding dHPLC chromatograms. An undigested PCR product is shown in the first lane for comparison. Mutant positive products are only digested once and therefore have a band at 277 bp. Wild type (W) FLT3 is digested twice with corresponding bands at 187 and 90 bp, as shown. (A) A minor $\Delta 836$ mutation detected by dHPLC but missed by *EcoRV*; (B) A heterozygous D835Y mutation detected by both dHPLC and *EcoRV*; (C) A minor D835E mutant detected by both dHPLC and *EcoRV*; (D) A D835V mutant where the *EcoRV* screen shows very little wild type FLT3 but the dHPLC trace is suggestive of only a small amount of heteroduplex; and (E) An unknown mutant with a shoulder on the main chromatogram peak that was negative by *EcoRV*.

mutation status be accurately and rapidly determined within 1-2 weeks of diagnosis. It is necessary therefore to develop sensitive and high throughput techniques that are capable of detecting these mutations.

The data presented in this chapter reports on a comparison 2 different techniques for the screening of a large cohort of younger adult AML patients for the presence of FLT3/TKD mutations. Using dHPLC, FLT3/TKD mutations in AML were detected in 161 of 1339 cases, 12%. This is considerably higher than the 7% reported in most other studies, although incidences up to 22% have been reported (Table 3.2). One possible explanation for the increased frequency of FLT3/TKD mutations detected in this study is that dHPLC is more sensitive than *EcoRV* restriction digest, a technique that had been used in the majority of previous studies. In a comparison of over 400 cases screened by both methods, there were no cases where dHPLC showed a normal chromatogram but the *EcoRV* digest suggested the presence of a mutation. Conversely, there were 17 cases which were *EcoRV* negative but dHPLC positive, demonstrating the superiority of the dHPLC technique.

One major limitation of restriction digest screening is that it will only detect mutations within a very specific region of DNA, usually 4-6 base pairs. Whilst the majority of FLT3/TKD mutations occur within codons 835 and 836, a number of mutations in the second kinase domain occur outside this region. One advantage of the dHPLC technique therefore, is that it will detect mutations across a wider region of DNA. In our cohort, there were 7 mutations which occurred outside of the *EcoRV* restriction digest site accounting for 4% of the mutant-positive patients. Other cohorts have reported a higher incidence of these mutations occurring between codons 840 and 842 (Jiang *et al*, 2004, Kindler *et al*, 2005, Spiekermann *et al*, 2002). Given the potential for the presence of a FLT3/TKD mutation to influence clinical management of patients with AML, the are clear advantages to the use of dHPLC.

A number of the mutations (n=13) detected by dHPLC but not by *EcoRV* occurred within the *EcoRV* digest site. As interpretation of *EcoRV* digestion is greatly dependent on the efficiency of the restriction digest, even using the modified approach adopted in this analysis, it can be extremely difficult to differentiate between an undigested band due to the presence of a lower level mutation or to incomplete digestion of FLT3/WT. Using dHPLC however, small heteroduplex peaks were detected in cases where the

EcoRV digest was negative or difficult to interpret (Figure 3.6). This observation highlights the sensitivity of the dHPLC technique for the detection of mutations that may only be present in a small proportion of the amplified alleles. The detection of such mutations may be important as somatic mutations only arising in a subclone of malignant cells may nevertheless be clinically or biologically important. Furthermore, whilst contamination by non-leukaemic cells is not a major problem in AML as the majority of samples contain a high percentage of leukaemic cells, many solid tumours are characterised by infrequent clonal malignant cells with a polyclonal stromal cell infiltrate. Sensitive techniques may therefore be required in order to detect mutations within the malignant cells in these cases.

Another advantage of dHPLC is that the chromatogram profile produced by different mutations was found to be specific and highly reproducible, in most cases enabling direct mutation identification from the WAVE pattern and confirmation using a mutation-specific restriction enzyme digest, without the need for sequencing (Figure 3.5). The distribution of mutations reported in this chapter is similar to that in previous studies, with the most frequent mutation, accounting for approximately 50% of mutations detected, converting an aspartate residue to a tyrosine at position 835 in the coding sequence (D835Y). There were three novel mutations which were detected in the present study, each in a single case. In one of these cases, a 16 base pair region of exon 20 was duplicated, the first report of a tandem duplication within this region of FLT3. Functional studies will be required in order to determine whether these mutations lead to constitutive activation of the FLT3 kinase.

Whilst the increased frequency of FLT3/TKD mutations in this study can partly be explained by the increased sensitivity of the dHPLC technique, this is not the only explanation, as even using *EcoRV* screening alone, the incidence would have been 10.8%, considerably higher than most other studies using the same method. There are a number of possible explanations for this. Firstly, although unlikely, it is possible that the modification to the *EcoRV* screening technique used in this study may have led to an increased sensitivity for the detection of lower level mutants. Secondly, it has previously been shown that FLT3/TKD mutations are associated with a leukocytosis at diagnosis (see chapter 4). It is more likely therefore, that sufficient material would have been available from FLT3/TKD⁺ cases than cases with FLT3/WT, although why this should differ between the present and other cohorts is unclear. Thirdly, as analysis of

the *EcoRV* digest was made in parallel with dHPLC analysis, it is possible that the dHPLC increased confidence in identifying difficult cases by *EcoRV*. Finally, variation in inclusion criteria between the studies, for example relating to the age of patients studied, may have influenced the overall incidence detected.

One potential disadvantage of the dHPLC technique is that it is dependent on the presence of heteroduplexes for the detection of mutant positive cases. Loss of the wild type allele has been reported for FLT3/ITDs (Kottaridis *et al*, 2001, Raghavan *et al*, 2005, Thiede *et al*, 2002, Whitman *et al*, 2001), if the same occurs in FLT3/TKD⁺ cases then these may be missed by dHPLC due to the lack of heteroduplexes. In practice, however, this is unlikely to be the case as even in samples where there is no FLT3/WT in any of the mutant cells, it is likely that there would be small amounts of 'contaminating' gDNA from FLT3/WT non-leukaemic cells sufficient to create some heteroduplexes and an abnormal dHPLC chromatogram. In the above study, of 1178 cases that were negative by dHPLC 269 were also screened by *EcoRV* digestion, all of these cases were WT by both techniques. There were only 2 cases detected by *EcoRV* screening with partial loss of the WT allele, both these cases were detected by dHPLC but had the appearance of lower level mutants (Figure 3.6). The measurement and significance of FLT3/TKD mutant levels are discussed in more detail in chapter 5.

In conclusion, the data presented in this chapter shows that dHPLC is a sensitive and rapid means of screening for FLT3/TKD mutations and is superior to *EcoRV* restriction enzyme screening. This information is significant to ongoing studies where FLT3/TKD mutation screening is required.

CHAPTER 4: CLINICAL CHARACTERISTICS AND PROGNOSTIC IMPACT OF FLT3/TKD MUTATIONS AND COMPARISON WITH FLT3/ITDs IN YOUNG ADULTS WITH AML

4.1 Introduction

In order to understand the clinical characteristics and outcome of patients with FLT3/TKD⁺ AML it is important to be able to compare and contrast this with the features of patients with FLT3/ITDs in order that the two types of mutation can be directly compared.

4.1.1 Clinical characteristics of AML patients with a FLT3/ITD

Following the discovery of FLT3/ITDs in patients with AML, an important question to address was whether the presence of a FLT3/ITD was associated with particular clinical phenotypes. Furthermore, in view of the limitations of current prognostic risk stratification in AML (see chapter 1), there was also considerable interest in whether FLT3/ITDs were a useful molecular marker for predicting response to therapy and long-term outcome in patients with AML. These questions have been addressed by screening large cohorts of patients for FLT3/ITDs. The results of these studies are summarised in Tables 4.1 and 4.2. The incidence reported for FLT3/ITDs in these cohorts is approximately 25% but varies considerably, with incidences reported between 13.2% and 38.5%, partly relating to heterogeneity in the age and cytogenetic status of the patients studied. There are, nevertheless, a number of findings that are reproducible across the studies. For example, in terms of the clinical features at presentation, the most striking finding is the association between the presence of a FLT3/ITD, a high WBC and high BM blast percentage. Consistent with this, a number of studies have also shown that FLT3/ITDs are associated with a high serum level of lactate dehydrogenase (LDH) at diagnosis, although this data is not available from the majority of studies. Some studies have suggested that the incidence of FLT3/ITDs is higher in younger compared to older adults with AML (Schnittger *et al*, 2002, Stirewalt *et al*, 2001). However, this has not been shown in any of the other series, and interestingly, paediatric AML cohorts have generally shown a lower incidence of FLT3/ITDs than those seen in adult patients (Kottaridis *et al*, 2003, Meshinchi *et al*, 2006).

Table 4.1: Studies of the clinical characteristics of adult AML patients with FLT3/ITDs

Reference	No.	Median Age (range)	ITD ⁺ (%)	Clinical Characteristics	FAB Type		Cytogenetics	
					Increased	Decreased	Increased	Decreased
(Kiyoi <i>et al</i> , 1997)	74 (APL)	NR	15 (20.3)	Leucocytosis (P<.001) High LDH (P=.04) Low fibrinogen (P=.04)	NR	NR	NR	NR
(Yokota <i>et al</i> , 1997)	112	NR	22 (19.6)	NR	NR	NR	NR	NR
(Kiyoi <i>et al</i> , 1999)	201 (non-APL)	NR	46 (22.9)	Leucocytosis (P<.001)	M2		NS	t(8;21)
(Abu-Duhier <i>et al</i> , 2000)	106	Mean 41.2 (15-74)	14 (13.2)	NR	NR		NS	Favourable
(Rombouts <i>et al</i> , 2000)	81	52 (16-88)	18 (22.2)	NS	NS		NS	NS
(Kottaridis <i>et al</i> , 2001)	854	41 (16-63)	227 (26.6)	Leucocytosis (P<.001) High BM Blasts (P<.001)	M3 (P=.004)	M0 (P=.02) M6 (P=.08) M7 (P=.04)	t15;17 (P<.001) NK (P<.001)	inv(16) (P<.001) 11q23 (P<.006) Complex (P<.001) Del(5q) (P.006) -5 (P.02); -7 (P=.01) t(8;21) (P<.001)
(Stirewalt <i>et al</i> , 2001)	140	67 (56-88)	41 (29.3)	Lower age (P=.03) High BM Blasts (P=.003) Leucocytosis (P<.001)	NS	NS	NS	Adverse (P<.001)
(Whitman <i>et al</i> , 2001)	82 (<i>de novo</i> , NK)	45 (20-59)	23 (28.0)	Leucocytosis (P=.01)	NS	NS	NR	NR
(Frohling <i>et al</i> , 2002)	523	NR (16-60)	119 (22.8)	NR	NR	NR	NK (P<.001) t(15;17) (P<.001)	inv(16) (P<.001) t(11q23) (P<.001) Complex (P<.001) t(8;21) (P<.001)
(Frohling <i>et al</i> , 2002)	224 (NK)	47 (16-60)	71 (31.7)	<i>De novo</i> AML (P=.002) Leucocytosis (P<.001) High BM Blasts (P=.002) High LDH (P<.001)	NR	NR	N/A	N/A
(Kainz <i>et al</i> , 2002)	53 (<i>de novo</i> , NK)	58 (NR)	16 (30.2)	Leucocytosis (P<.05) High LDH (P<.05)	NS	NS	N/A	N/A

Table 4.1: continued

(Kainz <i>et al</i> , 2002)	21 (APL)	52 (NR)	8 (38.1)	NS	NS	NS	NS	NS
(Noguera <i>et al</i> , 2002)	90 (APL)	NR	33 (36.7)	Leucocytosis (P<.001)	M3v (P<.001)	NS	BCR3 (P=.003)	NS
(Thiede <i>et al</i> , 2002)	979	NR	200 (20.4)	Leucocytosis (P<.001) High BM Blasts (P<.01)	NS	M6 and M4Eo (P<.05)	NK (P<.001) t(15;17) (P<.001) t(6;9) (P<.001)	Complex (P<.001) -7/7q- (P<.001) -5/5q- (P<.05)
(Schnittger <i>et al</i> , 2002)	1003	NR	234 (23.3)	Leucocytosis (P<.001) Younger age (P=.04) Females (P=.02) <i>De novo</i> AML	M3v M5b	M5a M4Eo	NK (P<.001) t(15;17) (P.01)	inv16 (P<.001) Complex (P<.001) -5/-7/7q- (P<.001) t(8;21) (P=.006) t(11q23) (P=.006)
(Boissel <i>et al</i> , 2002)	159	44 (15-65)	40 (25.2)	Leucocytosis (P=.002) Females (P=.04)	NS	NS	NK (P<.001)	t(8;21) (P<.001) inv(16) (P<.001)
(Moreno <i>et al</i> , 2003)	208	NR	36 (17.3)	Leucocytosis (P<.001)	NS	NS	NK (P=.04)	NS
(Shih <i>et al</i> , 2003)	107 (APL)	38 (2-76)	22 (20.6)	NS	M3v (P=.002)	NS	BCR-3 (P=.005)	NS
(Sheikhha <i>et al</i> , 2003)	80	52 (17-74)	8 (10.0)	Leucocytosis (P=.004)	M4	NS	NK	NS
(Jilani <i>et al</i> , 2003)	85	56 (18-84)	18 (21.2)	Leucocytosis (P<.001)	NS	NS	NS	NS
(Andersson <i>et al</i> , 2004)	109	70 (60-90)	20 (18.3)	Leucocytosis (P<.01)	M4 and M5 (P<.05)	NS	NS	NS
(Au <i>et al</i> , 2004)	82 (APL)	39 (7-87)	16 (19.5)	Leucocytosis (P<.001)	M3v (P=.01)	NS	NS	NS
(Chillon <i>et al</i> , 2004)	176	NR	34 (19.3)	NR (ITDs and TKDs grouped together)	NR	NR	NR	NR
(Gale <i>et al</i> , 2005b)	203 (APL)	37 (1-60)	69 (34.0)	Leucocytosis (P<.001)	M3v (P<.001)	NS	t(15;17) alone (P<.001) BCR3 (P=.002) RARA-PML expression (P=.01)	NS
(Wang <i>et al</i> , 2005)	143	39 (NR)	37 (25.9)	Leucocytosis (P<.001)	M3 and M5 (P.01)	NS	NK t(15;17)	NS

Table 4.1: continued

(Auewarakul <i>et al</i> , 2005)	256	NR	70 (27.3)	Leucocytosis (P<.001)	NR	NR	NR	NR
(Kuchenbauer <i>et al</i> , 2005)	170 (APL)	NR (17-83)	53 (38.4)	Leucocytosis (P=.003)	M3v (P<.001)	NS	BCR3 High PML-RARA expression level	NS
(Callens <i>et al</i> , 2005)	117 (APL)	43 (29-55)	45 (38.5)	Leucocytosis (P<.01)	M3v (P<.01)	NS	BCR2 and 3 (P<.05)	NS
(Mathews <i>et al</i> , 2007)	94 (APL)	29 (NR)	20 (21.3)	Leucocytosis (P=.07)	NS	NS	BCR3 (P=.01)	NS

NK indicates normal karyotype; APL, acute promyelocytic leukaemia; NS, not significant; NR, not reported.

Table 4.2: Studies of the clinical outcome of adult AML Patients with a FLT3/ITD

Reference	No.	Median Age (range)	No. Mutants (%)	CR	RD/DFS/EFS	RR	OS	Multivariate
(Kiyoi <i>et al</i> , 1999)	201	49 (15-85)	46 (22.9)	ND	DFS Reduced	NR	Reduced (P=.004)	Reduced DFS (P=.006) OS NS
(Kiyoi <i>et al</i> , 1999)	NR	<60 yrs only	NR	NR	NR	NR	NR	OS reduced (P=.008)
(Abu-Duhier <i>et al</i> , 2000)	106	Mean 41.2 (15-74)	14 (13.2)	NR	NR	NR	Reduced (P<.001)	NR
(Rombouts <i>et al</i> , 2000)	70	NR	17 (24.3)	Reduced (P=.03)	Reduced (P=.002)	NR	Reduced (P=.001)	NR
(Kottaridis <i>et al</i> , 2001)	854	41 (16-63)	227 (26.6)	NS	DFS and EFS reduced (P<.001)	Increased (P<.001)	Reduced (P<.001)	Increased RR (P<.001) DFS reduced (P<.001) OS reduced (P=.009)
(Stirewalt <i>et al</i> , 2001)	140	67 (56-88)	41 (29.3)	NS	NS	NS	NS	NS
(Frohling <i>et al</i> , 2002)	224 (NK)	47 (16-60)	67 (29.9)	NS	RD reduced (P=.007)	NR	Reduced (P<.001)	NR for ITD alone
(Noguera <i>et al</i> , 2002)	90 (APL)	NR	33 (36.7)	NS	NS	NS	NS	NS
(Thiede <i>et al</i> , 2002)	640 (Non-APL)	NR	111 (17.3)	NS	DFS Reduced (P=.03)	NR	Reduced (P.09)	NS
(Thiede <i>et al</i> , 2002)	254 (<i>de novo</i> , IR)	<60yrs	56 (22.0)	NS	Reduced (P=.007)	Increased (P=.008)	NS	NS
(Schnittger <i>et al</i> , 2002)	563	NR	126 (22.4)	NS	EFS reduced (P.007)	NR	NS	NR
(Kainz <i>et al</i> , 2002)	53 (<i>de novo</i> , NK)	58 (NR)	16 (30.2)	NS	DFS reduced (P=.02)	NR	Reduced (P=.003)	Reduced OS (P=.002)
(Kainz <i>et al</i> , 2002)	21 (APL)	52 (NR)	8 (38.1)	Improved (P=.05)	NS	NS	Improved (P=.06)	NR
(Boissel <i>et al</i> , 2002)	159	44 (15-65)	40 (25.2)	NS	NS	NS	NS	NS
(Moreno <i>et al</i> , 2003)	146	NR	28 (19.2)	NS	DFS reduced (P=.03)	NR	Reduced (P=.003)	NR
(Shih <i>et al</i> , 2003)	107 (APL)	38 (2-76)	22 (20.6)	NS	NS	NS	NS	NR
(Sheikhha <i>et al</i> , 2003)	80	52 (17-74)	8 (10.0)	NR	Reduced (P=.04)	NR	Reduced (P=.007)	NR
(Jilani <i>et al</i> , 2003)	85	56 (18-84)	18 (21.2)	NS	NS	NR	Reduced OS in <50yrs (P=.02)	NR
(Andersson <i>et al</i> , 2004)	109	70 (60-90)	20 (18.3)	NS	NS	NS	NS	NR
(Au <i>et al</i> , 2004)	82 (APL)	39 (7-87)	16 (19.5)	NS	Reduced (P=.05)	NR	NS	NS
(Gale <i>et al</i> , 2005b)	203 (APL)	37 (1-60)	69 (34.0)	NS	NS	NS	NS	NS
(Wang <i>et al</i> , 2005)	143	39 (NR)	37 (25.9)	Reduced (P=.01)	NR	NR	NR	NR

Table 4.2: continued

(Kuchenbauer <i>et al</i> , 2005)	170 (APL)	NR (17-83)	53 (38.4)	NR	NS	NR	Reduced (P=.09)	NS
(Callens <i>et al</i> , 2005)	117 (APL)	43 (29-55)	45 (38.5)	NS	NR	NS	Reduced (P=.09)	NR
(Auewarakul <i>et al</i> , 2005)	256	NR	70 (27.3)	NR	NR	NR	Reduced (P=.002)	NR
(Mathews <i>et al</i> , 2007)	94 (APL)	29 (NR)	20 (21.3)	NS	NS	NR	NS	NR

IR indicates intermediate risk; NK indicates normal karyotype; APL, acute promyelocytic leukaemia; NS, not significant; NR, not reported.

Correlation of FLT3/ITD status with FAB subtypes demonstrated that FLT3/ITDs are particularly common in patients with M3 (acute promyelocytic leukaemia, APL), more specifically the microgranular variant (M3v), as well as the monocytic leukaemias (M4 and M5a). Conversely, some studies have shown that FLT3/ITDs are uncommon in M4Eo, M5b, M6 and M7. Some, but not all studies, have observed an increased frequency of FLT3/ITDs in patients with *de novo* AML compared to those with secondary AML arising as a transformation from myelodysplasia.

Analysis of karyotype of the leukaemic blasts at diagnosis and correlation with FLT3/ITD mutation status is of considerable importance as this may provide biological insights into cooperating genetic events in AML. These analyses have shown that FLT3/ITDs are found at an increased frequency in patients with a normal karyotype (NK), a t(15;17) translocation and the rare t(6;9) translocation. Conversely, FLT3/ITDs are uncommon in patients with the core binding factor (CBF) leukaemias inv(16) and t(8;21), patients with a complex karyotype, patients with 11q23 (mixed lineage leukaemia [MLL]) abnormalities and patients with complete or partial deletion of chromosomes 5 and 7. In terms of cytogenetic risk groups, FLT3/ITDs are found at a reduced frequency in patients with favourable (excluding APL) and adverse cytogenetics and are more common in cases with intermediate cytogenetics.

4.1.2 Clinical outcome of patients with FLT3/ITD⁺ AML

With regards to clinical outcome, Kiyoi *et al* (1999) were the first group to screen a large cohort of adult patients with non-APL, *de novo* AML. They demonstrated an adverse outcome in FLT3/ITD⁺ patients, a finding that remained highly significant in multivariate analysis and that has been reproduced in most, but not all, studies (Table 4.2). Consequently, it is now widely accepted that FLT3/ITDs are an adverse prognostic marker predicting for reduced OS in comparison with FLT3/WT patients. Importantly, patients with an ITD achieve CR at a similar rate to those with FLT3/WT. The presence of a FLT3/ITD, however, predicts for a much higher RR, particularly in the crucial intermediate cytogenetic risk group where karyotype is not an informative prognostic marker as the majority of patients have a NK. For example, in the study from the department at UCL it was shown that in the intermediate risk group the RR at 5 years was 48% for FLT3/WT patients as opposed to 74% for FLT3/ITD⁺ patients (Kottaridis *et al*, 2001). In multivariate analysis in the MRC cohort of patients, the presence of a FLT3/ITDs was the single most powerful predictor of RR in young adults with AML.

This is particularly relevant for risk stratification as relapse remains the main cause of treatment failure in AML. At a biological level, the increased RR associated with FLT3/ITDs but lack of difference in CR rate is of interest. It suggests that, whilst the presence of a FLT3/ITD does not influence the chemosensitivity of the bulk of leukaemic cells, in patients who enter remission, residual leukaemic stem cells are less likely to be eradicated by post remission therapies in patients with a FLT3/ITD than those without a FLT3/ITD.

4.1.3 Clinical features and outcome of patients with FLT3/TKD⁺ AML

In view of the highly significant clinical associations of FLT3/ITDs in AML, following the discovery of FLT3/TKD mutations there was considerable interest in whether these mutations were associated with the same clinical features. The clinical features and prognostic implications of FLT3/TKD mutations reported in the literature are summarised in Tables 4.3 and 4.4. Like FLT3/ITDs, most studies have demonstrated that FLT3/TKDs are associated with a leucocytosis at diagnosis. A number of studies have also shown that FLT3/TKD mutations are less common in patients with secondary AML. The cytogenetic associations of FLT3/TKD mutations are less clear than for FLT3/ITDs. This may partly relate to the reduced frequency of FLT3/TKD mutations, necessitating the study of much larger cohorts of patients in order to have the power to demonstrate such associations. The largest studies reported to date, excluding the data presented in this chapter, have suggested that, like FLT3/ITDs, FLT3/TKD mutations are also observed at an increased frequency in patients with a NK (Bacher *et al*, 2008, Thiede *et al*, 2002). There was also an inverse correlation between the presence of a FLT3/TKD mutation and a complex karyotype or deletion of chromosome 5.

An area of considerable interest in the literature relates to the clinical significance of FLT3/TKD mutations in non-APL AML. As FLT3/ITDs may prove to be a clinically useful marker for risk stratification in AML, it is important to know whether FLT3/TKDs are associated with a similarly adverse prognosis. Many different groups have attempted to address this question but all have failed to show a statistically significant impact of a FLT3/TKD mutation on OS (Table 4.4). One group observed a trend for reduced OS ($P=.09$) associated with FLT3/TKDs in a study including 37

Table 4.3: Clinical characteristics of patients with FLT3/TKD mutations

Reference	No.	Median Age (range)	No. Mutants (%)	Dual TKD/ITD mutants (%)	Clinical Characteristics	FAB Type	Cytogenetics
(Abu-Duhier <i>et al</i> , 2001)	97	NR (16-85)	7 (7)	0 (0)	NS	NS	NS
(Yamamoto <i>et al</i> , 2001)	429	NR	30 (7)	1 (0.2)	NS	Increased: M5 (P=.02)	NS
(Thiede <i>et al</i> , 2002)	979	NR	75 (7.7)	17 (1.7)	Leucocytosis (P<.001) High BM Blasts (P<.01)	NS	Increased: NK (P<.01) Reduced: -5/5q- (P<.05)
(Moreno <i>et al</i> , 2003)	208	NR	20 (9.6)	4 (2.2)	NS	NS	NS
(Frohling <i>et al</i> , 2002)	224 (NK)	47 (16-60)	32 (14.3)	4 (1.8)	<i>De novo</i> AML (P=.002) High BM Blasts (P=.002)	NR	N/A
(Noguera <i>et al</i> , 2002)	90 (APL)	NR	7 (7.8)	NR	NR	NR	NR
(Sheikhha <i>et al</i> , 2003)	80	52 (17-74)	6 (7.5)	0 (0)	NS	NS	NS
(Shih <i>et al</i> , 2003)	107 (APL)	38 (2-76)	20 (18.7)	3 (2.8)	NS	NS	NS
(Shih <i>et al</i> , 2004)	120	42 (15-74)	13 (10.8)	NR	NS	NS	NR
(Andersson <i>et al</i> , 2004)	109	70 (60-90)	11 (10.1)	3 (2.8)	Leucocytosis (P=.07)	NS	NS
(Au <i>et al</i> , 2004)	82 (APL)	39 (7-87)	18 (22.0)	1 (1.2%)	NS	NS	NS
(Chillon <i>et al</i> , 2004)	135	NR	7 (5.2)	NR	NR	NR	NR
(Gale <i>et al</i> , 2005b)	203 (APL)	37 (1-60)	23 (11.3)	4 (2.0)	Leucocytosis (P=.001)	NS	NS
(Auewarakul <i>et al</i> , 2005)	256	NR	15 (5.9)	7 (2.7)	NS	NS	NS
(Kuchenbauer <i>et al</i> , 2005)	170 (APL)	NR (17-83)	8 (5.7)	1 (0.6)	NS	NS	NS
(Callens <i>et al</i> , 2005)	112 (APL)	43 (29-55)	22 (19.6)	8 (7)	Increased in males (P<.05)	NS	NS
(Mathews <i>et al</i> , 2007)	94 (APL)	29 (NR)	11 (11.7)	0 (0)	NS	NS	NS
(Whitman <i>et al</i> , 2008)	217 (NK)	47 (19-59)	16 sole TKD+ (7.4)	3 (1.4) excluded	Leucocytosis (P<.001) High BM blast % (P<.001)	NS	N/A
(Bacher <i>et al</i> , 2008)	3082	63 (18-92)	147 (4.8)	NR	Leucocytosis (P<.001) <i>De novo</i> AML (P=.01)	Increased: M3v (P=.02) M4 (P<.001) M5b (P<.001) Reduced: M2 (P<.001) M6 (P=.01)	Increased: NK Reduced: complex karyotype (P<.001)

NK indicates normal karyotype; APL, acute promyelocytic leukaemia; NS, not significant; NR, not reported.

Table 4.4: Clinical Outcome of AML Patients with a FLT3/TKD

Reference	No.	Median Age (range)	No. Mutants (%)	CR	DFS	RR	OS	Multivariate
(Abu-Duhier <i>et al</i> , 2001)	82	NR	7 (9)	NR	NR	NR	NS	NR
(Yamamoto <i>et al</i> , 2001)	201	NR	8 (4)	NS	NS	NS	NS	NR
(Thiede <i>et al</i> , 2002)	640 (Excluding APL)	NR	37 Sole TKD (5.8)	NS	NS	NR	Reduced (P=.09)	NS
(Thiede <i>et al</i> , 2002)	254 (<i>de novo</i> , intermediate risk)	<60yrs	20 Sole TKD (7.9)	NS	NS	NS	Reduced (P=.015)	NS
(Frohling <i>et al</i> , 2002)	224 (NK only)	47 (16-60)	28 sole TKD (12.5)	NS	NS	NS	NS	NR
(Moreno <i>et al</i> , 2003)	146	NR	14 (9.6)	NS	Reduced (P=.04)	NR	NS	NR
(Sheikhha <i>et al</i> , 2003)	80	52 (17-74)	6 (7.5)	NS	NS	NS	NS	NS
(Shih <i>et al</i> , 2003)	107 (APL only)	38 (2-76)	20 (18.7)	NS	NS	NS	NS	NR
(Shih <i>et al</i> , 2004)	120	42 (15-74)	13 (10.8)	NR	NR	NS	NS	NR
(Andersson <i>et al</i> , 2004)	109 (>60 yrs only)	70 (60-90)	11 (10.1)	NS	NS	NS	NS	NR
(Au <i>et al</i> , 2004)	82 (APL only)	39 (7-87)	18 (22.0)	NS	NS	NR	NS	NS
(Gale <i>et al</i> , 2005b)	203 (APL only)	37 (1-60)	19 TKD only (9.4)	Reduced CR (P=.04) Increased ID (P=.04)	NR	NS	Reduced (P=.05)	NS
(Auewarakul <i>et al</i> , 2005)	256	NR	15 (5.9)	NR	NR	NR	NS	NR
(Kuchenbauer <i>et al</i> , 2005)	170 (APL only)	NR (17-83)	8 (5.7)	NR	NS	NR	NS	NS
(Callens <i>et al</i> , 2005)	112 (APL only)	43 (29-55)	22 (19.6)	NS	NR	NS	NS	NR
(Mathews <i>et al</i> , 2007)	94 (APL only)	29 (NR)	11 (11.7)	NS	NS	NR	NS	NR

Table 4.4: continued

(Whitman <i>et al</i> , 2008)	217 (<i>de novo</i> , NK, <60)	47 (19-59)	16 sole TKD+ (7.4)	NS	Reduced (P=.01)	NR	NS	Reduced DFS (P=.02) OS NS
(Bacher <i>et al</i> , 2008)	1051	NR	69 (6.6)	NS	NS	NS	NS	NS

Three studies were published after completion of the work presented in this thesis and are included in the table for completion. NK indicates normal karyotype; APL, acute promyelocytic leukaemia; NS, not significant; NR, not reported.

TKD⁺ patients, excluding those with dual FLT3/TKD and FLT3/ITD mutations (Thiede *et al*, 2002). This study also performed a subgroup analysis of patients <60 years old with *de novo*, NK AML that included 20 FLT3/TKD⁺ patients. This subgroup analysis suggested that FLT3/TKDs were associated with an adverse OS (P.015) although, interestingly, there was no impact on RR or DFS. The uncertainty regarding the prognostic impact of FLT3/TKDs is partly due to the low incidence of the mutations such that relatively small numbers of mutant-positive patients have been investigated. In order to address this, a meta-analysis of four studies was performed which concluded that the outcome of FLT3/TKD mutations may be similar to that of FLT3/ITDs (Yanada *et al*, 2005). However this meta-analysis only included a total of 83 mutant positive patients, with consequently wide confidence intervals. Furthermore, whilst FLT3/TKD mutations were suggested to have an adverse DFS, no significant impact on OS was observed. Moreover, the amalgamation of these retrospective, observational studies with heterogeneous patient age and treatment protocols warrants a degree of caution in the interpretation of the data.

A definitive study to determine the prognostic significance of FLT3/TKD mutations is, therefore, important for the application of risk-adapted treatment in these patients, particularly in view of the debate regarding optimal therapy for FLT3/ITD⁺ patients (Gale *et al*, 2005a, Litzow 2005) and the current interest in molecularly targeted therapy with FLT3 inhibitors (Sternberg and Licht 2005). The clinical characteristics and prognostic impact of FLT3/TKD mutations were therefore examined in a large cohort of uniformly treated young adult AML patients with comprehensive clinical follow-up data available.

4.2 Patients, Materials and Methods

4.2.1 Patients

As detailed in chapter 3, 1339 adults with AML entered into either the UK MRC AML 10 (n=444) or AML12 (n=895) trials were screened for FLT3/TKD mutations. Demographics and cytogenetic data on the patients included in this analysis were available via the MRC Clinical Trial Unit and are shown in Table 4.5. Both these trials were also open to children, but the present analysis was only conducted on samples from patients ≥15 years of age. Median age at trial entry was 42 years and only 29 were over 60 years of age. The majority of patients had *de novo* AML (n=1246, 93%).

Details of FLT3/TKD mutation screening are described in chapter 3. The FLT3/ITD status on this cohort of patients was available as a result of previous work in the department at UCL (Gale *et al*, 2005a).

4.2.2 Therapy

The MRC AML 10 trial had two randomisations in patients <56 years of age. The first randomisation was between two different induction schedules. One consisted of 2 courses of DAT chemotherapy (daunorubicin [50 mg/m² by slow intravenous push on days 1, 3 and 5]; cytarabine [100 mg/m² 12-hourly by iv push on days 1 to 10 for the first course and 1 to 8 for the second course] and thioguanine [100 mg/m² 12 hourly, orally days 1-10 for the first course and 1 to 8 for the second course]). The other induction regimen was ADE (cytarabine [100 mg/m² 12-hourly by iv push on days 1 to 10 for the first course and 1 to 8 for the second course]; daunorubicin [50 mg/m² by slow intravenous push on days 1, 3 and 5] and etoposide [100 mg/m² by 1 hour intravenous infusion days 1 to 5]). Patients who entered CR then received consolidation with MACE (amsacrine [100 mg/m² by 1 hourly intravenous infusion days 1 to 5]; cytarabine [200 mg/m² iv by continuous infusion days 1 to 5] and etoposide [100 mg/m² by 1 hour intravenous infusion days 1 to 5]) and MiDAC (Mitoxantrone [10 mg/m² by short intravenous infusion days 1 to 5]; cytarabine [1.0 g/m² by short intravenous infusion days 1 to 5]). The second randomisation was open to patients who completed 4 courses of chemotherapy and compared high-dose therapy with autologous bone marrow transplant (BMT) rescue versus no further therapy. Patients who had an HLA-matched sibling donor were scheduled for allogeneic BMT after 4 courses of chemotherapy and were not randomised to receive autologous-BMT or not.

The MRC AML 12 trial also randomised patients between 2 different induction schedules. One consisted of 2 cycles of ADE chemotherapy as used in AML 10. The other induction regimen consisted of 2 cycles of MAE (mitoxantrone [12 mg/m² by intravenous infusion on days 1, 3 and 5]; cytarabine [100 mg/m² 12-hourly by iv push on days 1 to 10 for the first course and 1 to 8 for the second course]; and etoposide [100 mg/m² by 1 hour intravenous infusion days 1 to 5]). After the first course of induction therapy, patients were assigned into risk groups (good, standard or poor) on the basis of karyotype and response to the first course of induction therapy as detailed in chapter 1. Good or standard risk patients (patients who entered CR and who completed both courses of induction chemotherapy) received one course of consolidation therapy with

MACE (identical to AML 10 schedule). Following this, good risk patients were randomised to receive one of two options. One was a further course of consolidation chemotherapy (MiDAC, identical to the AML 10 schedule). The other was two courses of consolidation chemotherapy (ICE then MiDAC). Standard risk patients were also eligible for these two options but in addition were randomised between two further options, immediate BMT or ICE followed by BMT. Patients in the transplant groups received an allogeneic BMT if an HLA-matched sibling donor was available or autologous BMT if a donor was not available. Poor risk patients were treated on a MRC relapsed/refractory protocol.

4.2.3 End Points

CR was defined as a normocellular BM aspirate with normal trilineage maturation and less than 5% blasts without a requirement for regeneration of peripheral counts. In practice, 97% of patients who achieved the protocol prescribed definition of CR had peripheral regeneration of neutrophils to $1.0 \times 10^9/\text{L}$ and platelets to $100 \times 10^9/\text{L}$. Remission failures were classified by the clinicians as either partial remission (5%-15% blasts or less than 5% blasts but a hypocellular BM), resistant disease (RD) defined as greater than 15% blasts in the BM, or induction death (ID) i.e. related to treatment or hypoplasia. When a clinician's evaluation was not available, deaths within 30 days of trial entry were classified as IDs and all other failures to achieve remission as RD. OS was defined as the time from trial entry to death. For patients entering CR, DFS was the time from first CR to an event (death in first CR or relapse). RR was calculated as the cumulative incidence of relapse (CIR) for which death during CR was considered a competing risk.

4.2.4 Statistical methods

The Mantel Haenszel test for trend (for ordinal data) and Fisher's exact test (in 2 x 2 tables) or chi-squared tests (for larger tables) were used to test for differences in clinical and demographic data by FLT3/TKD positivity. Kaplan-Meier life-tables were constructed for survival data and were compared by means of the log rank test, with surviving patients in AML10 censored on 1st April 2004, and surviving patients in AML12 censored on 1st April 2005. Follow up was up to date for the vast majority of patients and the small number of patients lost to follow up were censored at the date they were last known to be alive. Analysis of time to event data was using standard logrank methods. Odds ratio (OR) plots, with tests for heterogeneity, were used to

investigate whether the prognostic relevance of FLT3/TKD differed between FLT3/ITD subgroups and different levels of FLT3/TKD mutant. Multivariate analysis was used to find the factors most closely associated with CR rate, and multivariate Cox models were used to analyse OS, DFS and CIR. Models were fitted using forward selection, with variables added to the model if they reached significance at the $P = .01$ level in univariate analysis. All P values are two-tailed. Statistical analysis was performed by Robert Hills (Department of Haematology, Welsh School of Medicine, Cardiff).

4.3 Results

4.3.1 Demographics and presenting clinical features of patients with a FLT3/TKD mutation

Details of the demographics and presenting clinical features of the 1339 patients stratified according to FLT3/TKD mutation status are given in Table 4.5. The presence of a mutation was not related to gender or age. Mutations were found in all FAB subtypes except M7, and there was some evidence that they occurred at an increased frequency in M4 ($P=.04$ for heterogeneity). FLT3/TKD mutations were less frequent in patients with secondary AML compared to *de novo* disease ($P=.007$). There was some evidence that the presence of a FLT3/TKD mutation may be correlated with a high WBC at presentation. The median WBC was $25.6 \times 10^9/L$ in mutant-positive patients versus $18.5 \times 10^9/L$ in WT patients ($P=.05$).

Information on karyotype was available in 1083 patients (Table 4.6). There was a high incidence of FLT3/TKD mutations in patients with inv(16) (13 of 55 patients, 24%, $P=.009$), but a low incidence in patients with t(8;21) although this was not statistically significant (5 of 78 patients, 6.4%, $P=.15$). There was also a low incidence in cases with adverse cytogenetics (3 of 107 patients, 3%, $P=.003$), in particular those with a complex karyotype (1 of 62 patients, 2%, $P=.007$).

4.3.2 Comparison of presenting clinical characteristics of FLT3/TKD⁺ and FLT3/ITD⁺ patients

In the total cohort of 1339 patients, 372 (27.8%) were FLT3/ITD⁺. Of 967 patients without a FLT3/ITD (FLT3/ITD⁻), 127 were FLT3/TKD⁺ (13%) compared to 34 of 372 FLT3/ITD⁺ patients (9%), a negative correlation that was statistically significant

Table 4.5 Clinical and demographic characteristics of 1339 AML patients

	Total (n)	FLT3/TKD ⁻ (% of total TKD ⁻)	FLT3/TKD ⁺ (% of total TKD ⁺)	Percent FLT3/TKD ⁺	<i>P</i>	Percent FLT3/ITD ⁺
Total	1339	1178	161	12%		28%
AML10	444	382 (32)	62 (39)	14%	.12	26%
AML12	895	796 (68)	99 (61)	11%		29%
Type of AML						
<i>De novo</i>	1247	1088 (92)	158 (98)	13%	.007	28%
<i>Secondary</i>	92	89 (8)	3 (2)	3%		25%
FAB type						
<i>M0</i>	38	35 (3)	3 (2)	8%	.04	8%
<i>M1</i>	215	196 (17)	19 (12)	9%		29%
<i>M2</i>	324	295 (26)	29 (19)	9%		24%
<i>M3</i>	226	194 (17)	32 (21)	14%		38%
<i>M4</i>	278	232 (21)	46 (30)	17%		30%
<i>M5</i>	130	112 (10)	18 (12)	14%		32%
<i>M6</i>	30	24 (2)	6 (4)	20%		7%
<i>M7</i>	15	15 (1)	0	0%		7%
<i>RAEB-t</i>	19	19 (2)	0	0%		5%
<i>Bilineage</i>	1	1 (<.5)	0	0%		100%
<i>Unknown</i>	63	55	8	0%		20%
Sex						
<i>Female</i>	667	581 (49)	86 (53)	13%	.3	29%
<i>Male</i>	672	597 (51)	75 (47)	11%		27%
Age (y)						
15-29	280	242 (21)	38 (24)	14%	.5	25%
30-39	297	264 (22)	33 (21)	11%		25%
40-49	387	339 (29)	48 (30)	12%		32%
50-59	346	308 (26)	38 (24)	11%		27%
60 or older	29	25 (2)	4 (2)	14%		38%
Median	42	42	41	N/A		N/A
WBC (x10⁹/L)						
<10	475	429 (37)	46 (30)	10%	.05	16%
10-19.9	196	172 (15)	24 (15)	12%		25%
20-49.9	276	240 (21)	36 (23)	13%		30%
50-99.9	190	161 (14)	29 (18)	15%		38%
100 or above	177	153 (13)	24 (15)	14%		48%
Unknown	25	23	2	8%		20%
Median	19.1	18.5	25.6	N/A		N/A
Cytogenetics						
Favourable	307	262 (27)	45 (35)	15%	.003	27%
Intermediate	669	590 (62)	79 (62)	12%		31%
Adverse	107	104 (11)	3 (2)	3%		7%
Unknown	256	222	34	13%		30%

P values relate to the incidence of FLT3/TKD mutations using Mantel-Haenszel test for trend in age, WBC count and cytogenetics, otherwise chi-squared or Fisher's exact test used for heterogeneity. N/A indicates not applicable.

Table 4.6: Incidence of FLT3/TKD⁺ patients in specific cytogenetic risk groups and subgroups

Cytogenetics	Total (n)	FLT3/TKD⁻ (n)	FLT3/TKD⁺ (n)	FLT3/TKD⁺ (%)	P	FLT3/ITD⁺ (%)
Favourable	307	262	45	15%		27%
t(15;17)	174	147	27	16%	.10	38%
t(8;21)	78	73	5	6%	.15	17%
inv(16)	55	42	13	24%	.009	7%
Intermediate	669	590	79	12%		31%
Normal	452	396	56	12%	.6	35%
del(7q)	24	22	2	8%	1.0	4%
11q23	30	28	2	7%	.6	3%
+8	92	78	14	15%	.3	24%
+22	16	13	3	19%	.4	6%
Adverse	107	104	3	3%		7%
Complex	62	61	1	1%	.007	5%
del (5q)	22	20	2	9%	1.0	0
-5	21	21	0	0%	.16	0
-7	42	40	2	5%	.2	2%
abn(3q)	33	32	1	3%	.17	15%
Unknown	256	222	34	13%		30%

P values are for Fisher exact test for the incidence of TKD mutations in each individual cytogenetic abnormality versus all other known karyotypes.

($P=.04$). There were a number of clinical features which were similar between FLT3/ITDs and FLT3/TKDs, for example, both were associated with a high WBC at diagnosis. In addition to this, both types of mutation were found at an increased frequency in patients with M4 AML and were uncommon in patients with adverse cytogenetics. Conversely, there were a number of features associated with FLT3/TKD⁺ cases that differed markedly from those of FLT3/ITD⁺ cases. For example, FLT3/TKD mutations were uncommon in patients with secondary AML whereas in this cohort the incidence of FLT3/ITDs did not differ significantly between *de novo* and secondary AML. Unlike FLT3/ITDs, which are particularly frequent in patients with t(15;17) and normal cytogenetics, the only group in which FLT3/TKD mutations were more common were cases with inv(16), a cytogenetic subgroup in which FLT3/ITDs are uncommon.

4.3.3 Relationship between the presence of a FLT3/TKD mutation and clinical outcome in patients with non-APL AML

APL patients are biologically distinct from non-APL cases of AML and are treated according to different regimens. The impact of mutant FLT3 status on clinical outcome in patients from this cohort with APL has already been published in previous work from the department at UCL (Gale *et al*, 2005b). Therefore, for the analysis of the impact of FLT3/TKD mutations on clinical outcome, APL patients have been excluded. Of 1107 non-APL cases, 127 were FLT3/TKD⁺. Outcome data was available on the whole cohort with a median follow up of 7.9 years (range 6 – 193 months). The CR rate for all 1107 patients was 85%. The presence of a FLT3/TKD mutation did not influence the rate of CR, ID or RD (Table 4.7). However, the presence of a FLT3/TKD mutation was associated with a reduced CIR at 5 years of 48% for FLT3/TKD⁻ versus 39% for FLT3/TKD⁺ cases ($P=.03$). Furthermore, the DFS was also improved at 35% and 48% ($P=.008$) for FLT3/TKD⁻ versus FLT3/TKD⁺ cases, which translated into an improved OS of 37% versus 53% respectively ($P=.002$). Of the total cohort of 1107 non-APL AML patients analysed for the impact of FLT3/TKD mutation status on clinical outcome, only 17 of the 127 mutant-positive patients received an allograft. This was an insufficient number for a robust analysis of the impact of allogeneic transplant according to FLT3/TKD mutation status and this analysis was therefore not performed.

4.3.4 Impact of cytogenetic subgroup on the prognostic impact of FLT3/TKD mutations

It is possible that the prognostic impact of FLT3/TKDs may be influenced by the underlying cytogenetic subgroup. Furthermore, given the high proportion of cases of *inv(16)* that are FLT3/TKD mutant-positive, it is possible that this unequal distribution may account for the favourable prognosis associated with FLT3/TKDs. To address this, the cohort was stratified according to cytogenetic risk group and specific cytogenetic abnormality. There was no heterogeneity or trend in the effect of a FLT3/TKD mutation on CIR (Figure 4.2A) or OS between the different subgroups (Figure 4.2B). Kaplan-Meier analysis also demonstrated that FLT3/TKD⁺ patients had an improved survival in the favourable and intermediate risk categories (Figure 4.3). As only 3 patients in the adverse cytogenetic risk group were FLT3/TKD⁺, survival for these patients is not shown.

Table 4.7: Clinical outcome in FLT3/TKD⁻ and FLT3/TKD⁺ non-APL AML patients

	TKD ⁻	TKD ⁺	OR (CI)	P	<i>Adjusted result: OR (CI)</i>
Initial Response to therapy					
CR	84%	88%	0.73 (0.44-1.21)	.2	0.80 (0.38-1.69); <i>P</i> .6
ID	6%	4%	0.68 (0.31-1.50)	.3	0.64 (0.19-2.15); <i>P</i> .5
RD	10%	8%	0.79 (0.42-1.48)	.5	0.94 (0.38-2.28); <i>P</i> .9
Outcome at 5 years					
CIR	48%	39%	0.75 (0.57-0.98)	.03	0.75 (0.53-1.07); <i>P</i> .11
DFS	35%	48%	0.74 (0.59-0.93)	.008	0.71 (0.52-0.97); <i>P</i> .03
OS	37%	53%	0.72 (0.58-0.89)	.002	0.71 (0.52-0.96); <i>P</i> .03

P values are for Mantel-Haenszel test for initial response, and log-rank test for long term outcomes. Adjusted results show results of multivariate analyses adjusted for significant variables in forward selection regression model. OR indicates odds ratio; CI, 95% confidence intervals.

4.3.5 Analysis of both FLT3/TKD and FLT3/ITD status

In view of the negative correlation between the presence of a FLT3/ITD and a FLT3/TKD mutation, outcome in a greater proportion of the FLT3/TKD mutant-negative cases would be influenced by the presence of a FLT3/ITD. Therefore, the impact of both types of mutation was examined. Of the 1107 non-APL AML patients, 723 (65%) had WT FLT3, 257 (23%) were FLT3/ITD⁺ only, 100 (9%) FLT3/TKD⁺ only and 27 (2%) had both mutations. The CIR at 10 years was 46% for FLT3 WT

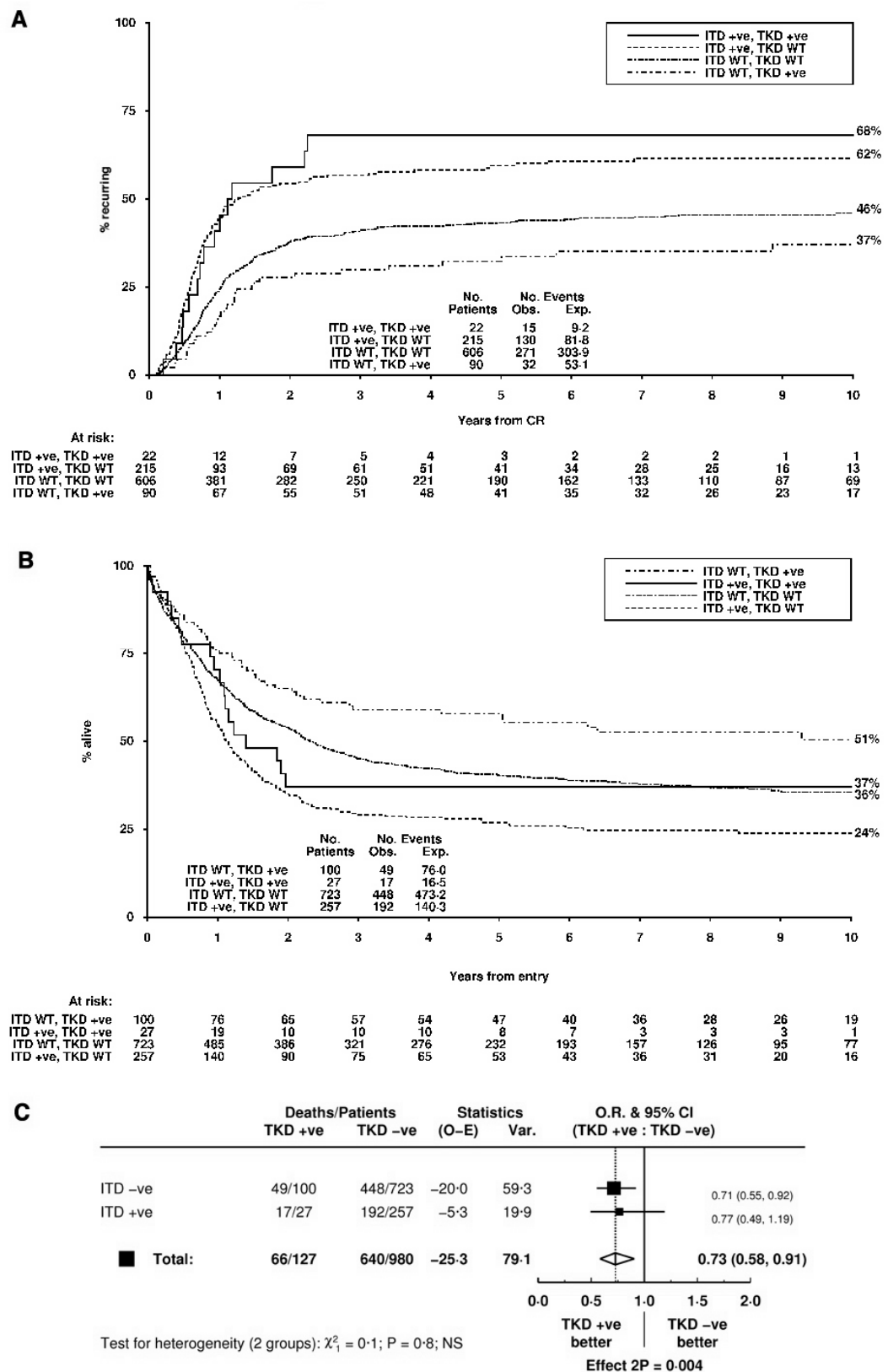


Figure 4.1. Clinical outcome for non-APL AML patients stratified according to FLT3/ITD and FLT3/TKD status. (A) Cumulative incidence of relapse, (B) overall survival, (C) Analysis of overall survival showing effect of FLT3/TKD status stratified by FLT3/ITD.

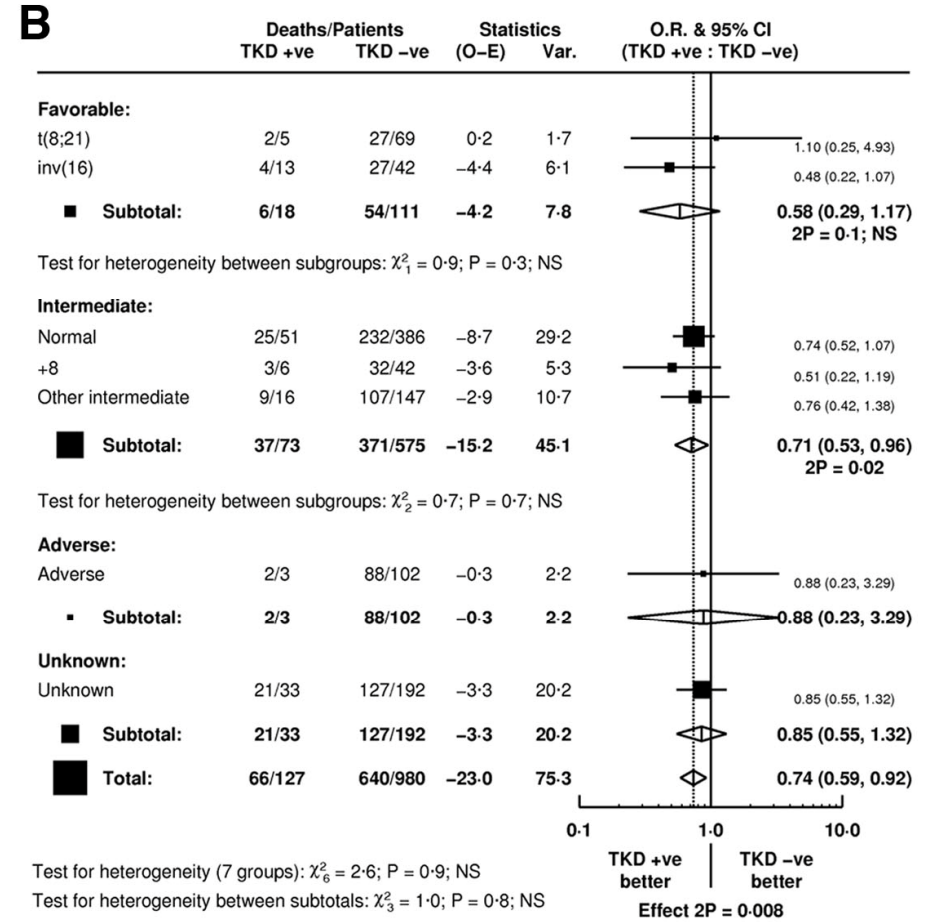
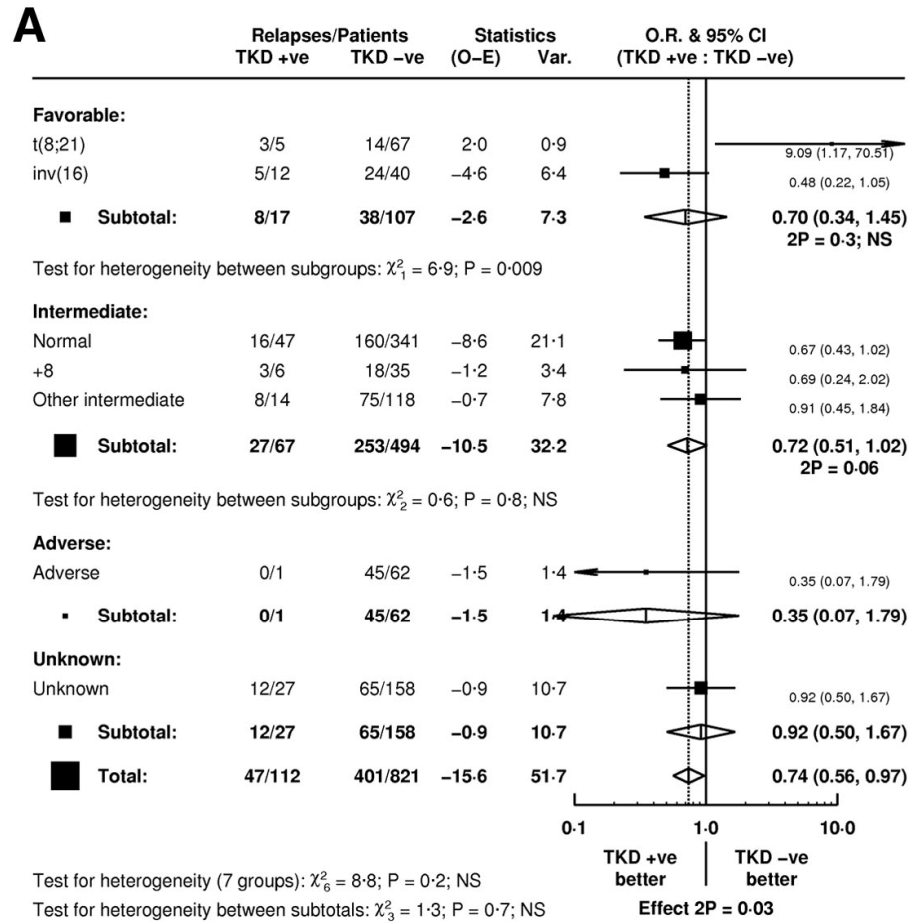


Figure 4.2. Cumulative incidence of relapse (A) and overall survival (B) stratified according to karyotype and cytogenetic category.

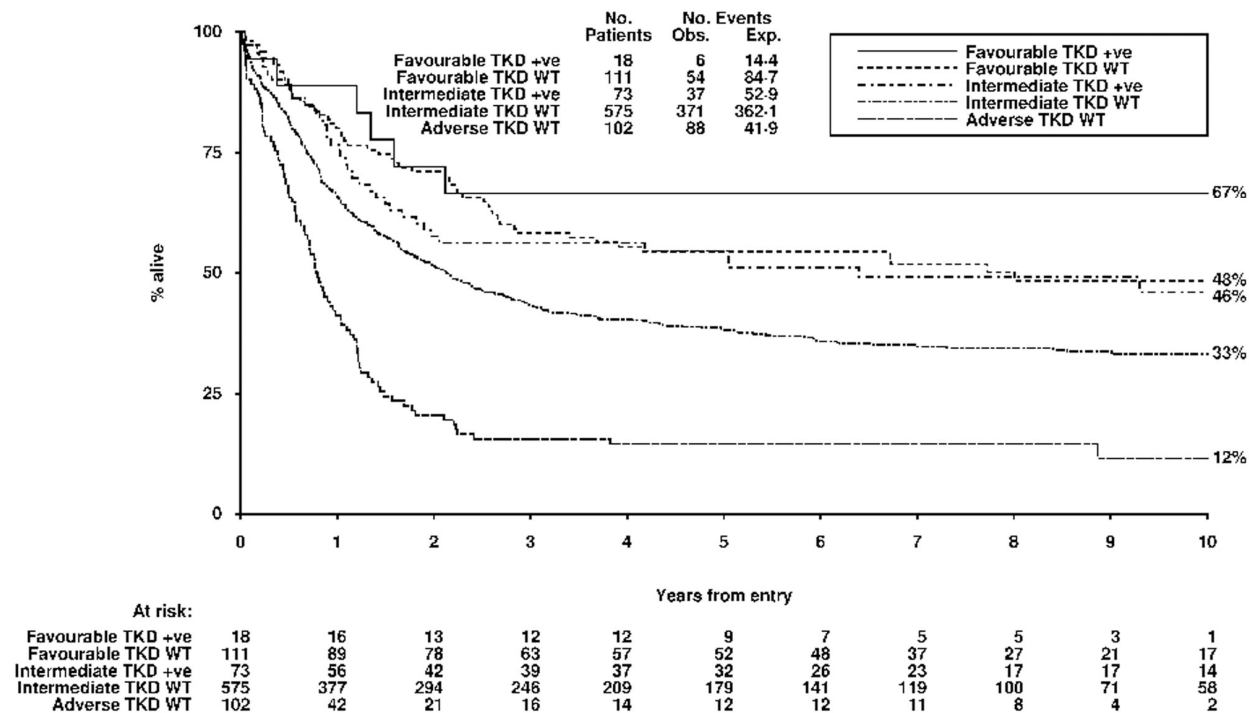


Figure 4.3. Overall survival in non-APL AML patients according to cytogenetic risk group. Kaplan-Meier curves stratified according to FLT3/TKD status and cytogenetic risk group. As only 3 patients with adverse cytogenetics carried a FLT3/TKD mutation this curve is not shown,

patients, 37% for FLT3/ITD⁻TKD⁺ patients, 62% for FLT3/ITD⁺TKD⁻ patients and 68% for FLT3/ITD⁺TKD⁺ patients (Figure 4.1A). OS for these groups was 36%, 51%, 24% and 37% respectively (Figure 4.1B). A direct comparison of FLT3/TKD mutations and FLT3/ITDs demonstrated a highly significant difference in CIR (OR = 0.45, 95% confidence intervals [CI] = 0.33-0.61, P<.001) and OS (OR = 0.53, CI = 0.41-0.69, P<.001). There was no evidence that the impact of a FLT3/TKD mutation on OS differed according to FLT3/ITD status (test for interaction, P=.8), although there were only 27 patients with both types of mutation (Figure 4.1C).

4.3.6 Multivariate analysis

A Cox multivariate analysis was performed, the variables considered being trial, cytogenetic risk group, age, sex, presentation WBC count, *de novo* or secondary AML, WHO performance status, and presence of a FLT3/ITD or TKD mutation. The prognostic impact of a FLT3/TKD mutation on OS was of borderline significance (OR = 0.71, CI = 0.52-0.96, P=.03). Other variables were more powerful, including presence of a FLT3/ITD (OR = 1.43, 95% CI = 1.19-1.72, P<.001), cytogenetics (OR = 1.80, 95% CI = 1.51-2.16, P<.0001), age (OR = 1.02, 95% CI = 1.01-1.02, P<.001), sex (OR = 0.79, 95% CI = 0.67-0.94, P=.007) and WHO performance status (OR = 1.13, 95% CI = 1.04-1.22, P=.003). The point estimate for the reduction in CIR associated with a FLT3/TKD mutation was of similar magnitude to the reduction in OS (OR = 0.75, 95% CI = 0.53-1.07, P=.13). The only variables predicting for increased CIR were the presence of a FLT3/ITD (OR = 1.82, 95% CI = 1.46-2.27, P<.001) and cytogenetic risk group (OR = 1.70, 95% CI = 1.35-2.13, P<.001). The OR and CI for clinical outcome analyses of FLT3/TKD mutations adjusted in a multivariate analysis including the above variables are shown in Table 4.7.

4.4 Discussion

The data presented in this chapter describe the associations of FLT3/TKD mutations with clinical characteristics and outcome of a large cohort of uniformly treated young adults with AML. As the clinical phenotype of FLT3/ITDs has already been well described in the literature (Tables 4.1 and 4.2), one of the main questions for this analysis was whether FLT3/TKDs were clinically distinct from or associated with a similar phenotype to FLT3/ITDs. There were a number of similarities observed between the two types of

mutation. For example, like FLT3/ITDs, FLT3/TKDs were associated with a leucocytosis at presentation. Furthermore, both mutations were infrequent in cases with complex cytogenetics or t(8;21), although the latter was not significant for FLT3/TKDs. The inverse correlation between the concurrent presence of both types of mutation could also be interpreted as suggesting that they confer similar biological effects to the cell and there is little advantage, therefore, for the 2 mutations to occur together.

A number of observations, however, suggest that the two different types of FLT3 mutation are clinically distinct. Firstly, they were associated with different underlying genetic events, and this had been noted in some, but not all, previously reported cohorts (Table 4.3). For example, with regards to cytogenetic subgroups, an increased frequency of FLT3/TKD mutations was noted in cases with inv(16), unlike FLT3/ITDs which were uncommon in this subgroup. Other cohorts have not examined sufficient numbers of FLT3/TKD⁺ patients to demonstrate such associations, although some have observed a trend for increased numbers of FLT3/TKD mutants associated with inv(16) (Care *et al*, 2003, Libura *et al*, 2003, Thiede *et al*, 2002). It has also been suggested that cases of FLT3/TKD⁺ with inv(16) are restricted to those with variant CBFbeta-MYH11 fusion transcripts (Libura *et al*, 2003), although this data was not available in our analysis. A second difference between ITDs and TKDs in our cohort was in the reduced frequency with which FLT3/TKDs were detected in secondary compared to *de novo* AML, which was not observed for FLT3/ITDs. Other cohorts, however, have observed a lower frequency of FLT3/ITDs in patients with secondary AML (Table 4.1). Finally, FLT3/ITDs have been shown in a number of studies to be found at a very high incidence in t(6;9) translocated cases of AML (42/55 cases, 76.4%) whereas no FLT3/TKD mutations were detected in 51 patients screened (Thiede *et al*, 2007).

The different cooperating genetic events associated with FLT3/ITDs and FLT3/TKDs may be a consequence of distinct mechanisms underlying the formation of each type of mutation, perhaps reflecting variable susceptibilities of the target cell of transformation to different types of DNA damage. Alternatively, the two types of mutation may lead to distinct intracellular events downstream of the activated receptor, with consequences for cellular phenotype and cooperation with other genetic events. The distinct patterns of gene expression that have been reported in primary leukaemic samples containing FLT3/ITDs

and TKD mutations (Neben *et al*, 2005) would support both explanations. It is, however, noteworthy that *in vitro* analysis in a cell model system transfected with the different types of mutant found that differential signaling was induced by the two mutations (Choudhary *et al*, 2005b). Moreover, in a mouse model transplanting retrovirally-transduced BM progenitors, the phenotype differed significantly according to the type of mutation transplanted (Grundler *et al*, 2005). Mice transplanted with a FLT3/ITD developed a lethal oligoclonal myeloproliferative disorder, those with an FLT3/TKD an oligoclonal lymphoproliferative disorder. The signalling events resulting from the two types of mutation are discussed in more detail in Chapter 7.

It is possible, therefore, that the differential association of ITDs and TKDs with inv(16) cases relates to differences in cooperating biological pathways. However, despite the rare association between FLT3/ITDs and inv(16) in patients with AML, it is interesting that FLT3/ITDs have recently been shown to cooperate with inv(16) to generate a leukaemic phenotype in a mouse model (Kim *et al*, 2007).

The most striking difference between FLT3/ITDs and FLT3/TKDs in our cohort of patients was the opposing impact on clinical outcome; the 10 year OS for FLT3/TKD⁺ patients was 51% compared to 24% for FLT3/ITD⁺ patients (Figure 4.1B). This association of FLT3/TKD mutations with improved long-term survival was unexpected, but the effect was robust and remained significant in a multivariate analysis. It is likely to be attributable to a reduced RR in FLT3/TKD⁺ patients (CIR at 10 years = 37% compared to 62% in FLT3/ITD⁺ patients) (Figure 4.1A). There was no association of a FLT3/TKD mutation, nor a FLT3/ITD (Kottaridis *et al*, 2001) with attainment of CR. This suggests that any leukaemic progenitors which remain post induction chemotherapy may be more chemo-sensitive if they are FLT3/TKD⁺ and, therefore, more likely to be eradicated. These results are in contrast to those of previous studies. A recent meta-analysis of the major studies concluded that FLT3/TKD mutations are associated with inferior DFS, although there was no significant evidence of TKD mutation adversely affecting OS (Yanada *et al*, 2005). The differences from our study may relate to heterogeneity of patient inclusion criteria, treatment schedules, length of follow up or the play of chance owing to the relatively small number of mutant-positive patients in previous studies. The clinical outcome data in the present study was based on 127 mutant-positive, uniformly treated patients with

comprehensive clinical follow-up data compared with a total of 83 heterogeneously treated patients in the meta-analysis.

The finding of different clinical outcome for two types of mutation in the same protein is surprising and raises some interesting biologic question regarding the mechanism(s) underlying this difference. Both types of FLT3 mutation lead to constitutive activation of the receptor in *in vitro* studies (Kiyoi *et al*, 1998, Yamamoto *et al*, 2001) and are thought to lead to loss of auto-inhibition of the kinase activity. Mutations in the TKD are thought to alter the configuration of the activation loop in a manner similar to that of ligand-induced conformational changes, allowing increased access of ATP and substrates to the kinase, whereas the tandem duplications are thought to disrupt the interaction between the JM domain and the activation loop which normally stabilises the kinase in its inactive configuration (Gilliland and Griffin 2002b, Griffith *et al*, 2004). The consequence of these different mechanisms may be quantitatively and/or qualitatively quite variable, for example, they may lead to altered substrate specificities downstream of the kinase (Songyang *et al*, 1995). Precisely how this may influence outcome is unclear. It is also possible that the difference in clinical outcome observed between the two mutant types is not a consequence of differential downstream signaling events but instead reflects the type of mutagenic event giving rise to the FLT3 mutation or the inherent susceptibility of different leukaemic stem cells to different types of DNA damage.

As the genetic and epigenetic events that underlie AML become understood in more detail, there is a drive towards developing a molecular classification for this disease. For example, the current WHO classification of AML includes distinct subgroups on the basis of karyotype abnormalities such as t(15;17) and inv(16) (Harris *et al*, 1999). This classification may be relevant to the study of FLT3 mutations as there is some evidence the clinical impact of FLT3/ITDs may differ between APL and non-APL cases of AML (Gale *et al*, 2005b). In the present analysis we tested for heterogeneity of the prognostic impact of FLT3/TKD mutations between different cytogenetic groups. Whilst no heterogeneity was detected, even with over 1000 patients studied, the present analysis was inadequately powered to detect such differences. There was a suggestion that the presence of a FLT3/TKD mutation conferred a favourable prognosis within the inv(16) subgroup, although this did not reach statistical significance (OR 0.48, CI = 0.22 – 1.07, P=.1, Figure

4.2B). Such a subgroup analysis needs to include other cooperating mutations within this group of patients such as FLT3/ITDs, RAS and c-Kit mutations. Determining the prognostic impact of FLT3 mutations in CBF leukaemias will therefore require considerable expansion of the present cohort of patients to adequately address this question.

As discussed in chapter 1, 40-50% of young adult patients with AML have an NK and there is particular interest in the use of molecular markers to stratify patients with NK AML into different risk categories (Mrozek *et al*, 2007). Understanding how these different mutations interact to impact the clinical phenotype is of considerable importance for this classification. Collecting sufficient numbers of patients for a robust analysis is the major limitation in such studies with regards to the less frequent genetic events such as MLL-PTDs, CEBP/ α , PTPN-11, AML-1 and WT-1 mutations (see chapter 1). A more frequent interaction does occur between FLT3 mutations and the recently described mutations of the nuclear chaperone protein NPM1 (Falini *et al*, 2005, Falini *et al*, 2007). In the NK group of patients, both FLT3/ITDs and FLT3/TKDs are associated with NPM1 mutations in the majority of cases. A recent analysis of the cohort of patients used in the studies presented here demonstrated that FLT3/ITDs are associated with an adverse prognosis in both NPM1⁺ and NPM1⁻ patients (Gale *et al*, 2007). Likewise, the favourable prognosis associated with NPM1 mutants was independent of FLT3/ITD status. An analysis of the interaction between FLT3/TKD mutations and NPM1 mutations is warranted as it is possible that the observed favourable prognostic impact of FLT3/TKD mutations relates to the frequent presence of NPM1 mutations in FLT3/TKD⁺ patients. However, a recent study has suggested that the presence of a FLT3/TKD confers additional favourable prognostic impact in patients with a NPM1 mutation, although this was of borderline significance (Bacher *et al*, 2008).

The results presented in this chapter have important implications for the treatment of patients carrying FLT3 mutations, particularly as there are new opportunities for the specific treatment of AML patients with aberrant FLT3 signaling using small molecule inhibitors targeting FLT3 (see chapter 6). Several FLT3 inhibitors are now available and are being assessed in combination with conventional chemotherapy in phase 3 clinical trials. If the different types of FLT3 receptor mutants have different downstream effects, then the effects of FLT3 inhibitors might also differ. Furthermore, in some centres, patients

with FLT3/ITDs are being considered for allogeneic transplantation in first remission (Litzow 2005), but in the absence of other poor prognostic factors, this might not be appropriate in patients with FLT3/TKD.

CHAPTER 5: THE IMPACT OF FLT3/TKD MUTANT LEVEL ON CLINICAL CHARACTERISTICS AND OUTCOME OF YOUNG ADULTS WITH AML

5.1 Introduction

Analysis of FLT3/ITD mutations has shown that the relative level of mutant and WT alleles varies widely in AML (Gale *et al*, 2007). Furthermore, FLT3/ITD mutant level has been shown to correlate with clinical characteristics and outcome (see below). Interestingly, FLT3/TKD mutation screening by dHPLC also revealed marked variability in the amount of heteroduplex that was present in FLT3/TKD⁺ cases (see Chapter 3), most likely reflecting similar variability of FLT3/TKD mutant levels. However, the clinical analysis described in the previous chapter stratified patients solely according to the presence or absence of a FLT3/TKD mutation. It is possible that heterogeneity within the group of FLT3/TKD⁺ patients may influence their clinical characteristics and prognosis. Therefore, in view of the biological and clinical significance of FLT3/ITD mutant level, the relative mutant level in FLT3/TKD⁺ cases is also worthy of further exploration.

5.1.1 Biological basis for different FLT3/ITD mutant levels

Quantification of mutant level in FLT3/ITD⁺ cases of AML has shown a very wide range of mutant level from 1% of total FLT3 alleles to greater than 90%. Study of the distribution of different mutant levels in our cohort of FLT3/ITD⁺ patients showed that they could be classified into three main groups (Gale *et al*, 2007). These are defined here as low level mutants accounting for 1-24% of FLT3 alleles, high level mutants with 25 to 50% mutant alleles and very high level mutants with greater than 50% mutant alleles.

The high level mutants are likely to represent cases where the majority of cells being heterozygous for the mutation. A low level of mutation, however, suggests that less than half the cells used to make the gDNA sample were heterozygous for the mutation. There are two possible explanations for this observation (Figure 5.1). Whilst it is possible that contamination by non-leukaemic cells in the gDNA sample has diluted the number of FLT3 mutant alleles, both clonality analysis and study of NPM1 mutation level suggested that, in

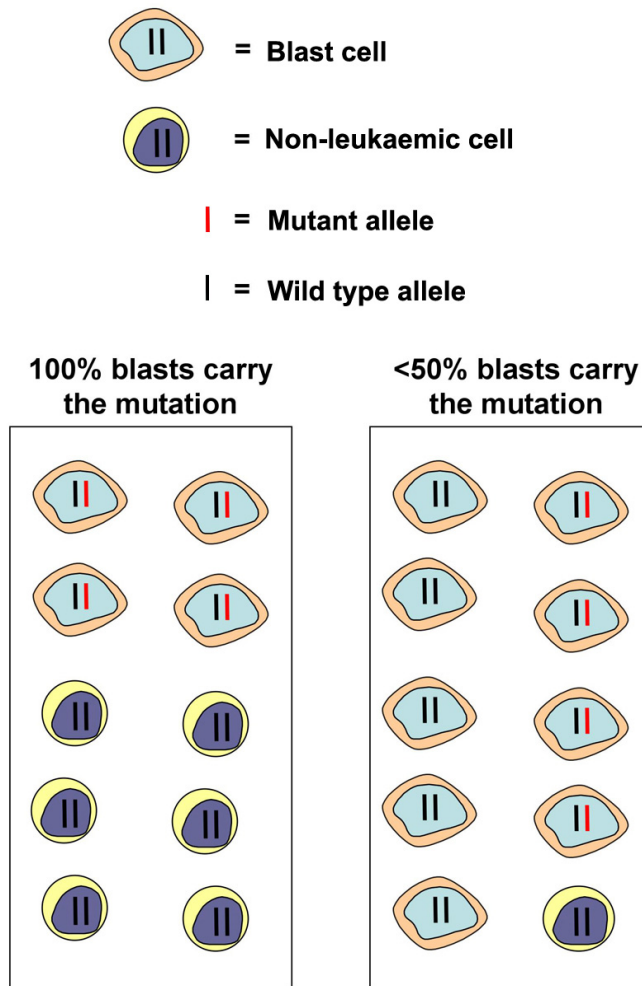


Figure 5.1: Possible explanations for a low level mutation accounting for 20% of alleles.

the majority of low level cases, this explanation was unlikely (Gale *et al*, 2007). It appears therefore, that in a proportion of cases with a low mutant level, FLT3/ITDs are only present in a minority of the leukaemic blasts.

Whilst AML in young adults is a clonal disorder originally derived from a single cell, it is also clear that a number of different hits are required for the development of AML (see Chapter 1). The accumulation of different genetic events is likely to be temporally separated as it is improbable that these hits would occur simultaneously in the same cell. A clonal population of AML blast cells may, therefore, actually consist of a heterogeneous mix of various subclones with different genetic damage. Some of these genetic events are

likely to be essential for the development of the leukaemia. However, it is also possible that some of these hits are not essential for the development of the leukaemia but may nevertheless influence the leukaemic phenotype, for example by conferring a selective advantage to a subclone of cells (see Figure 5.2). Consequently, study of the mutant level may provide valuable insights into this phenomenon as hits that are present in the majority of blast cells are likely to have occurred as an early or primary event in leukaemogenesis and those that are only present in a minority of the cells were acquired as a late or secondary event.

A very high mutant level (greater than 50%) suggests that, at least in a proportion of the cells, the WT allele has been lost. As shown in Figure 5.3 there are two possible explanations for this. Firstly, the FLT3/WT allele may be deleted. Secondly, the WT allele may be lost due to mitotic recombination event leading to a homozygous FLT3/ITD. Early studies using microsatellite analysis showed that loss of the FLT3/WT allele was due to loss of heterozygosity at the FLT3 locus (Whitman *et al*, 2001), and that deletion of this region on chromosome 13 was not apparent by FISH (Thiede *et al*, 2002). Subsequently, genome wide single nucleotide polymorphism arrays have provided strong evidence that a mitotic recombination event is, indeed, the most likely explanation for cases with a very high mutant level (Fitzgibbon *et al*, 2005, Raghavan *et al*, 2005).

5.1.2 Clinical impact of FLT3/ITD mutant level

A number of studies have examined the clinical impact of different FLT3/ITD mutant levels (see Table 5.1). Given the heterogeneity of the patients included in these studies, as well as different methods of analysis of the mutant level, direct comparison is difficult. However, there are a number of findings that are consistent between these studies. Patients with a very high level of mutation, consistent with loss of the WT allele, are associated with a higher WBC and a particularly adverse prognosis. The first study to report this (Whitman *et al*, 2001) observed loss of the FLT3/WT allele in 8 of 23 FLT3/ITD⁺ cases in a cohort of 85 young adults with NK AML. The DFS at 1 year was 71% for FLT3/WT patients, 51% for those with a heterozygous FLT3/ITD and 17% for patients with an ITD and loss of the FLT3/WT allele. This observation has been confirmed in larger cohorts of adult (Gale *et al*, 2007, Thiede *et al*, 2002) and paediatric AML (Meshinchi *et al*, 2006).

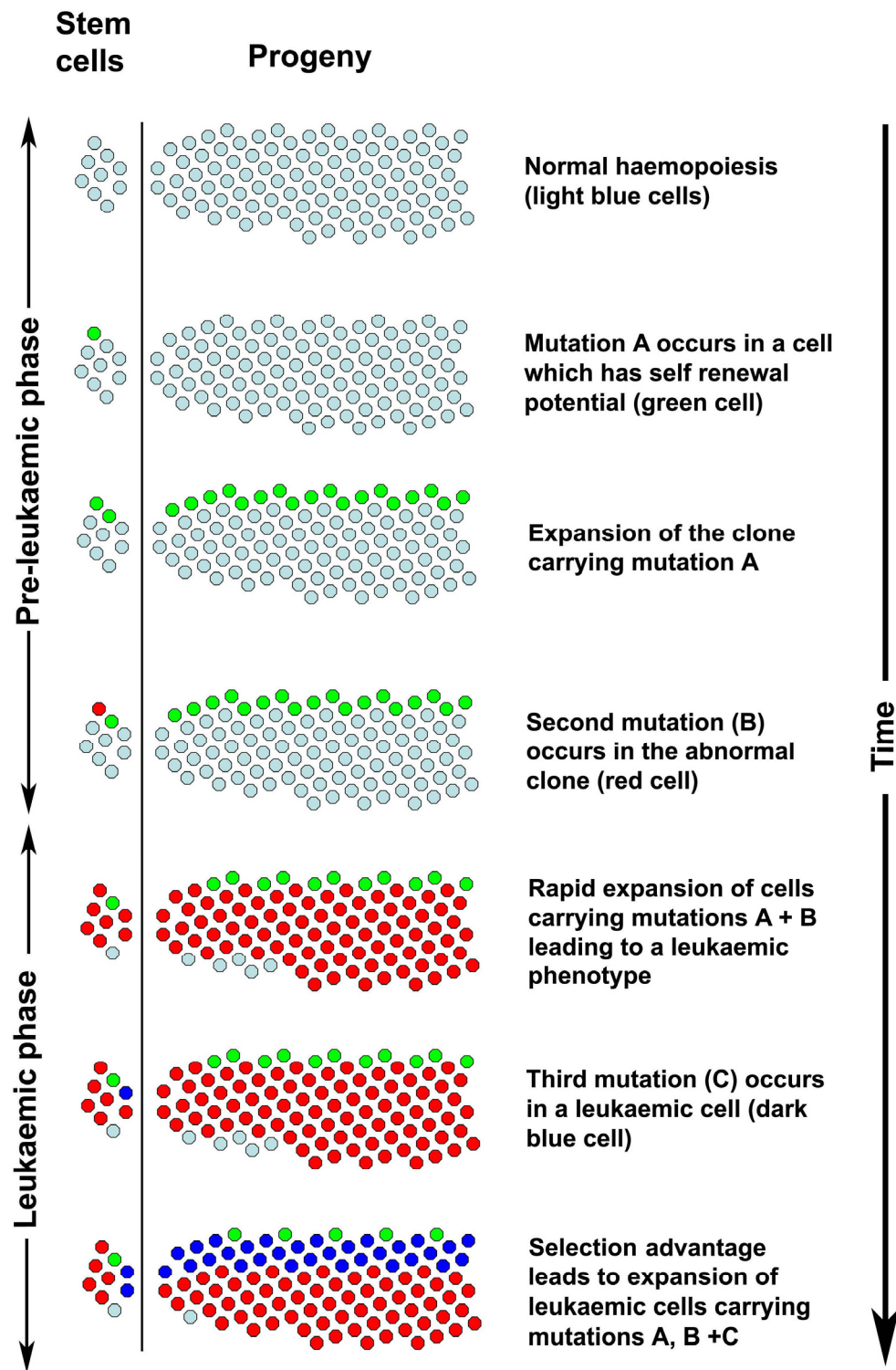


Figure 5.2: Diagrammatic representation of early and late mutations in leukaemogenesis. In this example, mutations A and B would be quantified as high level mutants and mutation C as a low level mutant.

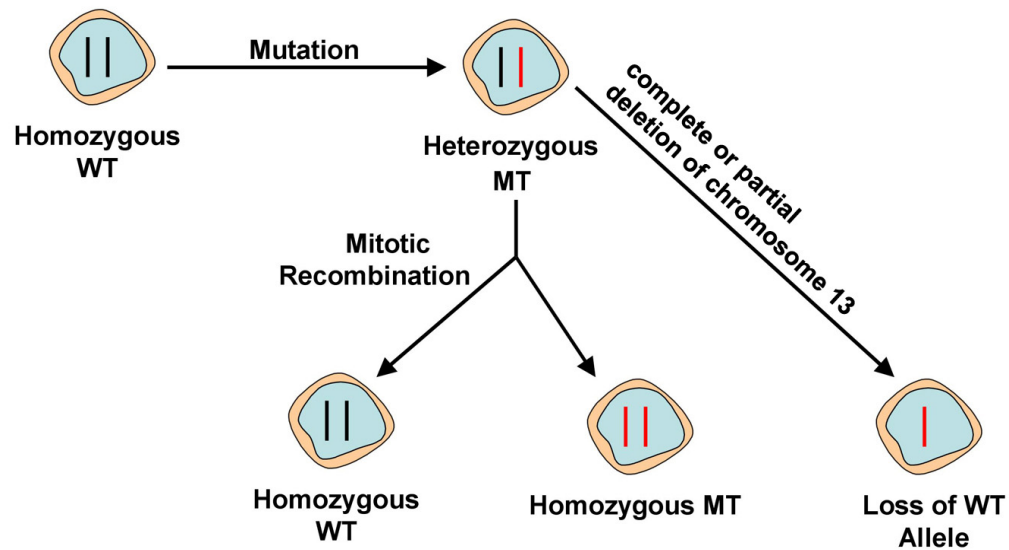


Figure 5.3: Possible explanations for high level FLT3 mutants

The prognostic impact of low level mutations is less clear. The study from UCL suggested that patients with a low level FLT3/ITD mutation had an adverse outcome that was similar to those with an high level of mutation (Gale *et al*, 2007). However, other studies have suggested that lower level FLT3/ITD mutants may have a clinical outcome not dissimilar to FLT3/WT patients and the adverse prognosis associated with ITDs relates only to those with high level mutants (Meshinchi *et al*, 2006, Thiede *et al*, 2002, Whitman *et al*, 2001). However, as shown in Table 5.1, these studies used different ‘cut-offs’ for low and high level mutants.

5.1.3 Paired presentation and relapse studies of FLT3/ITD mutations

Further insights into the biology of FLT3/ITDs have been gained though the study of paired samples from presentation and first relapse (Kottaridis *et al*, 2002, Nakano *et al*, 1999, Schnittger *et al*, 2004, Shih *et al*, 2002). These studies have shown that FLT3/ITDs are lost at relapse in approximately one quarter of cases. This is important as it limits the utility of FLT3/ITDs as a molecular marker for MRD analysis. This provides further evidence that, at least in a proportion of cases, FLT3/ITDs occur as a late event in leukaemogenesis. In cases where the mutant is retained at relapse, the mutant level is often increased, either

from a low to high level or from high to very high level. This suggests that the subclone of leukaemic cells carrying a heterozygous ITD or homozygous ITD had a proliferative advantage and/or increased chemoresistance compared to FLT3/WT or heterozygous FLT3/ITD⁺ cells respectively.

Table 5.1: Association of FLT3/ITD Level with Clinical Characteristics and Outcome in non-APL AML

Reference	No. (inclusion criteria)	No. Mutants (%)	Cut-Off for high level	No. high level Mutants	Clinical characteristics	Clinical outcome	Multivariate
(Whitman <i>et al</i> , 2001)	82 (NK, <60 yrs)	23 (28%)	Visual estimation ITD>WT	8 of 23 (35%)	Higher WBC (P=.01)	Reduced DFS (P=.03) and OS (P=.008)	NR
(Thiede <i>et al</i> , 2002)	254 (<i>de novo</i> , intermediate risk, <60yrs)	59 (23%)	>44%	29 (49%)	Higher WBC and BM blasts (NR)	Reduced DFS (P=.004) and OS (P=.006)	Reduced DFS and OS (P<.01)
(Jilani <i>et al</i> , 2003)	85 (18-85 years)	18 (21%)	Visual estimation ITD>WT	5 of 18 (28%)	Higher WBC (NR)	Reduced OS (NR)	NR
(Meshinchi <i>et al</i> , 2006)	630 (<i>de novo</i> , 0.5-19 years)	77 (12%)	>29%	54 (70%)	NS	Reduced PFS (P=.001)	Reduced PFS (P<.001)
(Gale <i>et al</i> , 2007)	1425 (non APL, young adults)	354 (26%)	>50%	53 (15%)	Higher WBC (P<.001)	Increased RR (P<.001) Reduced DFS and OS (P<.001)	Increased RR (P<.001) and Reduced OS (P<.001)

NR indicates not reported and PFS, progression free survival.

In view of the biological and clinical information gained from these studies of FLT3/ITD mutant level, it is possible that additional insights would also be gained by the study of FLT3/TKD mutation level. FLT3/TKD mutant level has been previously studied in a small cohort of patients, revealing marked heterogeneity in the relative mutant level between 1%

and 71% (Shih *et al*, 2004). However, this was a small study including only 13 FLT3/TKD⁺ patients at diagnosis, precluding any detailed study of clinical characteristics or outcome. In order to address this issue, FLT3/TKD mutant level was quantified in the cohort of FLT3/TKD⁺ patients described in Chapter 4 and results correlated with clinical characteristics, outcome and, where possible, mutation status at relapse.

5.2 Methods

5.2.1 Characteristics of the cohort studied

The FLT3/TKD mutant level was studied in 127 FLT3/TKD⁺ patients from the cohort of 1107 non-APL AML patients. Treatment schedules, definition of end-points and statistical methods used are detailed in chapter 4.

5.2.2 Quantification of FLT3/TKD mutants by semi-quantitative PCR and denaturing polyacrylamide gel electrophoresis

The relative level of mutant was quantified using semi-quantitative PCR and restriction enzyme digestion. The mutant identification method described in section 3.2.4 was modified by reducing the cycle number to 25 and end-labelling primers 20F or 20R2 with γ -[³²P]-ATP (3000 Ci/mmol, Amersham Pharmacia Biotech, Little Chalfont, UK). Samples were digested according to the known mutation (Table 3.3) and electrophoresed through denaturing polyacrylamide gels (7 M urea, 6% polyacrylamide cross-linker ratio 37.5:1, 1 x Tris-borate-EDTA). The gels were transferred to 3MM Chr chromatography paper, dried and exposed to a Fuji BAS-IIIi imager plate overnight. The plates were visualised using a Molecular Dynamics Typhoon PhosphorImager and quantified using ImageQuant 5.2 software (Molecular Dynamics) (Figure 5.4). The level of mutant FLT3 is expressed as a percentage of the total signal.

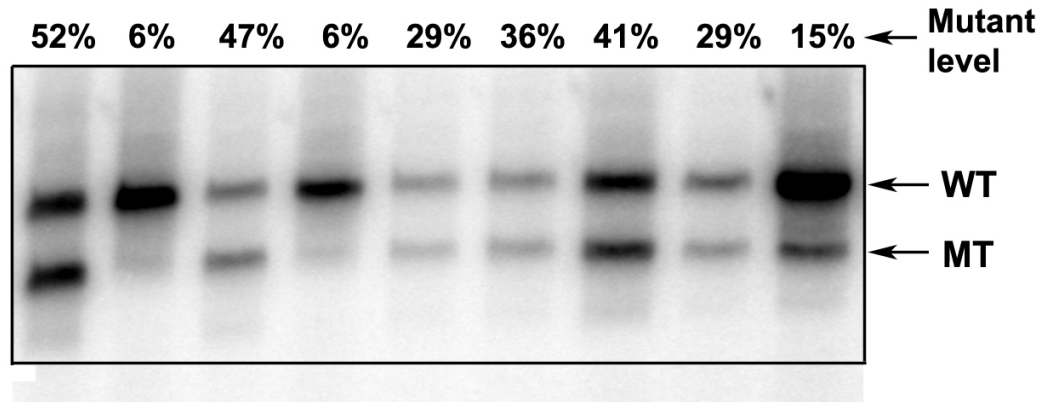


Figure 5.4: Analysis of the level of 9 D835V mutations using a radiolabelled 20F primer and *HincII* digestion.

5.2.3 Quantification of FLT3/TKD mutants by fragment analysis

The quantification of FLT3/TKD mutant level was also optimised for fragment analysis. The relative level of mutant was quantified using PCR as described in chapter 3, but with a fluorescently labeled primer and reduced cycle number (30 cycles) followed by fragment analysis on the CEQ8000 DNA Genetic Analysis System. A new primer (20R4: 5'-GCCCAAGGACAGATGTGATGC-3'), 171 bp further downstream of codon 835 than 20R, was used in order to generate longer PCR products for optimal analysis using this technique. For deletions and insertions, undigested PCR products were analyzed; for other mutations, products were first digested with either a mutation-specific restriction enzyme (Figure 5.5) or *EcoRV*. The level of mutant FLT3 is expressed as a percentage of the total signal. A small number of mutant samples (n=6) were quantified using both techniques in order to confirm that comparable results were obtained.

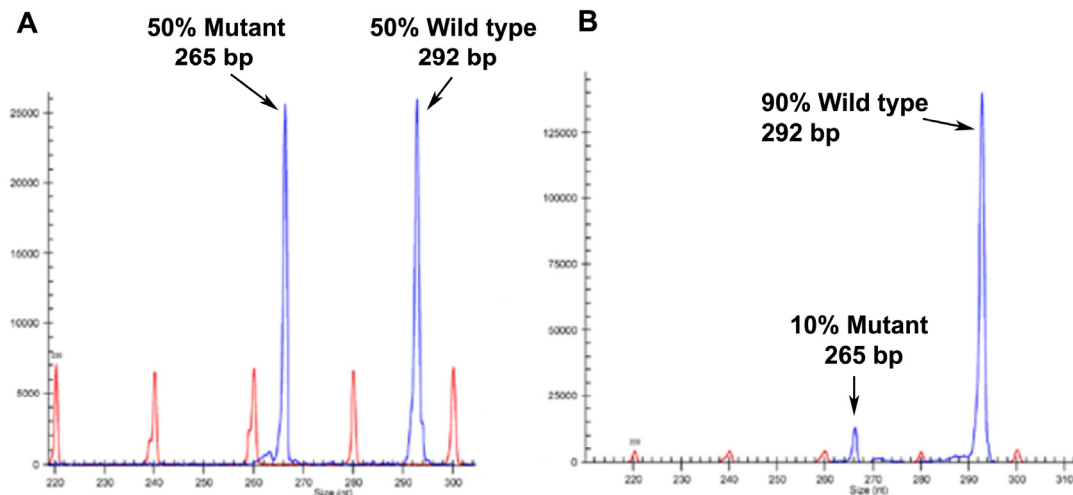


Figure 5.5: Analysis of two D835Y mutants by fragment analysis after *Cla*I digestion.

(A) A high level mutant accounting for 50% of total FLT3 alleles and, (B) a low level mutant accounting for 10% of total FLT3 alleles.

5.3 Results

5.3.1 Distribution of mutant level

The relative mutant level as a percentage of total FLT3 alleles was quantified in 115 known FLT3/TKD⁺ samples using labelled PCR products and size separation, after mutation-specific or *Eco*RV restriction enzyme digestion if required. The distribution of the mutant level is shown in Figure 5.6. Quantification was not performed in 12 cases, either because a mutation-specific restriction digest was not available (4 cases), the mutation had not been identified (3 cases) or due to lack of material (5 cases), and therefore these samples were scored by visual estimation of the dHPLC chromatogram. Mutants were arbitrarily classified as high level if the mutant level was greater than the median of 25%, or low level when less than or equal to this. Of note, only two patients (2% of all FLT3/TKD mutants) showed clear evidence of biallelic mutation or loss of WT alleles, with 88% and 93% mutant level respectively. Nine other FLT3/TKD mutants had levels of between 51 and 70%, thus only 11 cases (9%) had a mutant level >50%. This is a lower frequency of very high level mutations than for FLT3/ITDs where these account for approximately 15% of all mutants (Gale *et al*, 2007). Therefore, for FLT3/TKDs, there was an insufficient number of

patients with a mutant level >50% for analysis as a separate category and they were included with the high level cases.

Of the total cohort of 127 mutant-positive cases, a high level of mutant was present in 62 (49%), i.e. 6% of all patients in the cohort, and the median mutant level was 43% (range, 26% - 93%). A low level of mutant was present in 65 cases (51%), i.e. 6% of all patients, with a median level of 6% (range, 1%-25%). In the five cases with two mutants, four had one major mutant of high level plus a minor mutant of very low level (approximately 2% of total), in the remaining case both mutants were lower level (less than 25% of total), this latter case was classified as a low level mutant.

5.3.2 Clinical characteristics according to FLT3/TKD mutant level

Low level FLT3/TKD⁺ patients had a similar WBC to FLT3/WT patients with median WBCs of $23 \times 10^9/L$ and $24 \times 10^9/L$ respectively. High level mutants, however, were associated with a high WBC (median $49 \times 10^9/L$; $P=.002$). There was no evidence of heterogeneity between high and low level FLT3/TKD mutants for trial, age, sex, performance status, cytogenetic category, *de novo* versus secondary AML or FAB type.

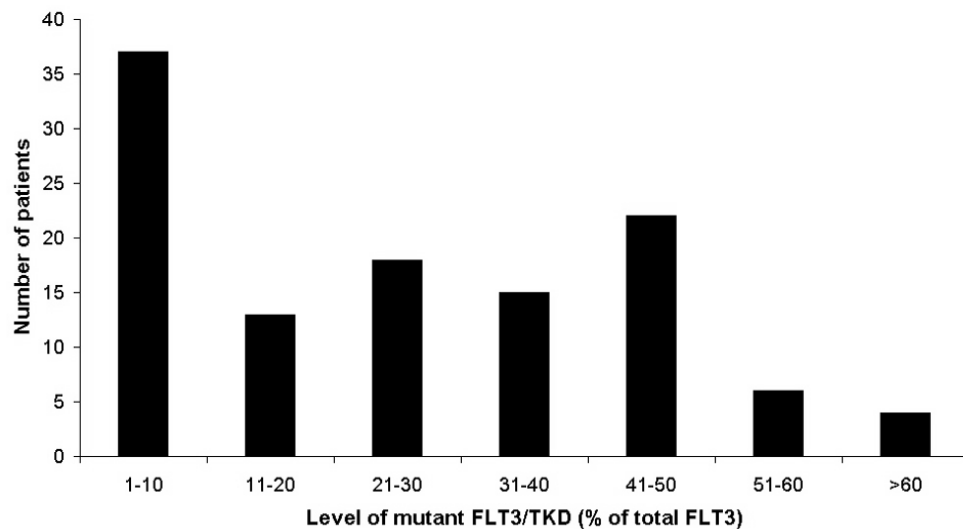


Figure 5.6. Distribution of the relative FLT3/TKD mutant level in 115 cases.

5.3.3 Distribution of FLT3/TKD and FLT3/ITD mutations according to mutant level

Analysis according to the presence or absence of a mutation showed a weak negative correlation between the presence of a FLT3/ITD and a FLT3/TKD mutation (see Chapter 4). Analysis according to the level of mutation, however, showed a highly significant negative correlation between the presence of a high level FLT3/ITD and a high level FLT3/TKD mutation (Table 5.2, $P < .001$). In fact, there were no cases where a high level FLT3/ITD and FLT3/TKD were present in the same sample.

Table 5.2: Distribution of FLT3/TKD and FLT3/ITD mutations according to mutant level

	ITD WT	ITD Low	ITD High	ITD V.High	Total
TKD WT	724 (88%)	72 (80%)	138 (95%)	46 (94%)	980
TKD Low	42 (5%)	13 (14%)	7 (5%)	3 (6%)	65
TKD High	57 (7%)	5 (6%)	0	0	62
Total	823	90	145	49	1107

For this analysis, an identical cut off was used for FLT3/ITDs and FLT3/TKDs. Low level mutants less than or equal to 25%, high level mutants 26-50% and very high level mutants (ITDs only) greater than 50%.

5.3.4 Clinical outcome according to FLT3/TKD mutant level

Proportional hazards regression analysis found significant evidence for a trend for higher FLT3/TKD levels to be associated with better survival ($P = .008$), however, there was no natural cut-off of mutant FLT3/TKD level that acted as an independent predictor of outcome. In view of this, for the analysis of clinical outcome, the median mutant level of 25% was again used as a cut off. In the total cohort of 1107 non-APL AML patients, including FLT3/ITD⁺ cases, there was no evidence of any impact of FLT3/TKD mutant level on the rate of CR, ID or RD (Table 5.3). Patients with a high level FLT3/TKD mutant, however, had an improved CIR and OS compared to patients with a lower level of mutation, whose outcome was not dissimilar to that of FLT3/TKD⁻ patients (Table 5.3). The CIR at 10 years was 50% for WT patients, 50% for patients with a lower level FLT3/TKD mutant and 37% for patients with a higher level mutant (Figure 5.7A). The OS at 10 years was 33%, 37% and 59% respectively (Figure 5.7B). This improved OS

remained significant in a cox multivariate analysis (OR = 0.74, 95% CI = 0.61-0.91, P=.004), although the difference was only of borderline significance for CIR (OR = 0.80, 95% CI = 0.64-1.00, P=.05).

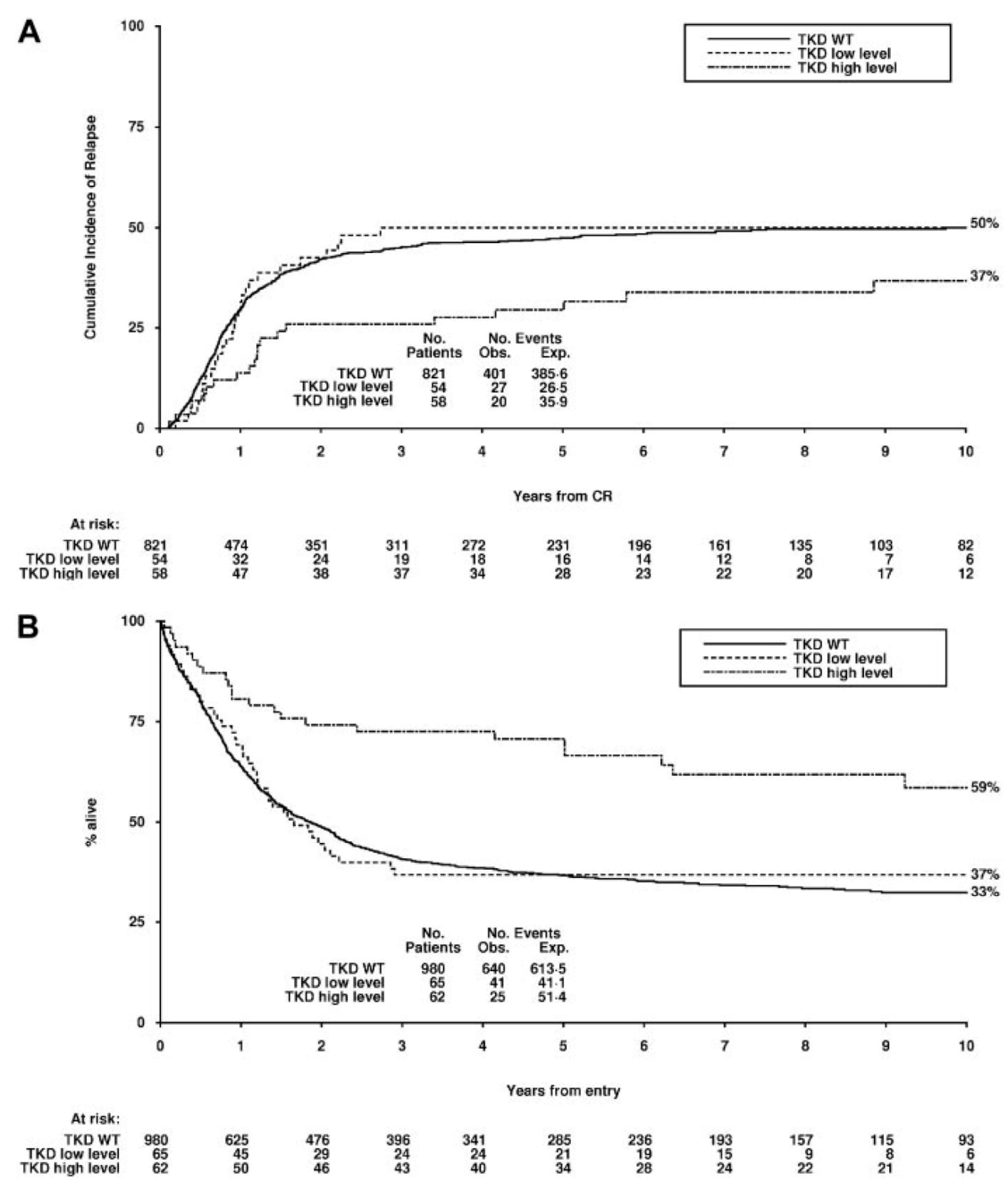


Figure 5.7: Clinical outcome for non-APL AML patients stratified according to FLT3/TKD mutant level. (A) CIR and (B) OS. High level mutations, more than 25% of FLT3 alleles; low level mutations, 25% of FLT3 alleles or less.

5.3.5 Paired presentation and relapse samples from FLT3/TKD⁺ patients

DNA samples at the time of relapse were available from 9 of the 51 FLT3/TKD⁺ cases who relapsed. Four cases with low mutant level (2 D835Y, 1 Δ836 and 1 unknown) and two cases with high mutant level (2 D835Y) all lost their mutation at relapse. Three high mutant level cases relapsed with the same mutation (2 D835Y, 1 Δ836) at a similar level.

5.4 Discussion

Analysis of FLT3/ITD mutant level has provided important biological insights into the pathogenesis of AML. Furthermore, stratification according to FLT3/ITD mutant level has helped to refine the prognostic information gained from mutation screening for detection of the presence or absence of the mutation alone. At the time of this analysis, however, similar studies had not been carried out for FLT3/TKD⁺ mutations.

Table 5.3: Clinical outcome in FLT3/TKD⁻ and FLT3/TKD⁺ non-APL AML patients

	TKD WT	TKD low	TKD high	OR (CI)	Adjusted result: OR (CI)
Initial Response					
CR	84%	83%	94%	0.72 (0.49-1.06); <i>P</i> .07	0.74 (0.44-1.29); <i>P</i> .3
ID	6%	6%	2%	0.65 (0.33-1.28) <i>P</i> .17	0.57 (0.22-1.47); <i>P</i> .2
RD	10%	11%	5%	0.78 (0.49-1.24); <i>P</i> .3	0.87 (0.48-1.57); <i>P</i> .6
Outcome at 5 years					
CIR	48%	50%	30%	0.78 (0.64-0.94); <i>P</i> .01	0.80 (0.64-1.00); <i>P</i> .05
DFS	35%	35%	60%	0.77 (0.65-0.91); <i>P</i> .003	0.78 (0.64-0.95); <i>P</i> .01
OS	37%	37%	71%	0.74 (0.63-0.87); <i>P</i> .004	0.74 (0.61-0.91); <i>P</i> .004

P values relating to TKD⁻ versus TKD⁺ analysis are for Mantel-Haenszel test for initial response and log-rank test for long term outcomes. *P* values relating to TKD⁻ versus TKD low versus TKD high analysis were determined using logistic regression analysis. Adjusted results show results of multivariate analyses adjusted for significant variables in forward selection regression model.

The data presented in this chapter reports on FLT3/TKD mutant level in 127 FLT3/TKD⁺ non-APL AML cases. The distribution of mutant levels was different to that of FLT3/ITDs. Cases with a very high level of mutation were less common for FLT3/TKD mutations (9%)

than for FLT3/ITDs (approximately 15%). Furthermore, only 2 (2%) cases had FLT3/TKDs mutant levels greater than 75% compared with 8% for FLT3/ITDs. Conversely, low level mutants were more frequent for FLT3/TKDs, accounting for approximately 50% of mutant positive patients compared with 29% of FLT3/ITDs. Very low level mutants, accounting for less than 5% of FLT3 alleles, suggesting that the mutation is present in less than 10% of cells, accounted for 22 of 127 FLT3/TKD mutants (17% of all mutants) compared with approximately 6% of FLT3/ITDs. As higher mutant levels are likely to correlate with mutations that are acquired as earlier events in leukaemogenesis, these data would suggest that FLT3/TKDs occur more frequently as late or secondary events compared with FLT3/ITDs.

These data differ from previous studies of FLT3/TKD mutant level. In a small cohort of 13 cases where FLT3/TKD mutant level was measured, 5 (38%) very high level mutants were present, with mutant levels between 54% and 71% (Shih *et al*, 2004). This study, however, did confirm the frequent presence of low level FLT3/TKD mutants, accounting for 6 of the 13 cases (46%). In another study, published subsequent to the present analysis, mutant level was quantified in 14 FLT3/TKD⁺ young adults with NK, *de novo* AML (Whitman *et al*, 2008). The authors reported that the mutant level ranged from 54% to 100%, with 8 patients having 100% of the FLT3 alleles mutated, suggesting that loss of the FLT3/WT allele is much more common for FLT3/TKDs than FLT3/ITDs in their cohort. The explanation for such a striking difference is not clear and is unlikely to be due to methodology as the authors report that they used an identical method to that used in the present analysis.

Stratification of our cohort according to mutant level and clinical characteristics at presentation showed that high level mutants were associated with a high WBC at presentation, whereas low level mutants had a WBC that was similar to that of FLT3/WT patients. These findings are similar to those reported for FLT3/ITDs (Gale *et al*, 2007). It is possible, therefore, that if a mutation is only present in a subclone of the leukaemic cells, this is not sufficient to lead to a leucocytosis for the population as a whole. There was also a highly significant inverse relationship between the presence of a high level FLT3/TKD and high level FLT3/ITD mutation, which never occurred together. Therefore, although FLT3/ITD and FLT3/TKD ‘dual mutant’ cases can be detected, at a biological level these

data would suggest that the two different mutations are unlikely to occur in the same leukaemic cell. This may relate to different mechanisms of mutagenesis for FLT3/ITDs and FLT3/TKDs. Alternatively, it may be that a leukaemic cell carrying one type of FLT3 mutation does not gain any additional proliferative advantage by acquiring the different type of mutation and is, therefore, not selected for.

Clinical outcome was also correlated with the level of the FLT3/TKD mutation, an analysis not made in previous studies. Favourable outcome was associated with FLT3/TKD⁺ patients with a high level of mutant. For example, patients with a high level mutant had an OS at 10 years of 59% compared to 37% in patients with a lower level mutant and 33% in patients without an FLT3/TKD mutation (Figure 5.7B). This difference remained significant in multivariate analysis (P=.004). This suggests that quantitative differences in the level of mutation, reflecting the proportion of leukaemic cells that carry the mutation, may be biologically important for the leukaemic population as a whole, for example, in determining the response to therapeutic agents. The only other study to have addressed this issue failed to demonstrate any significant association between FLT3/TKD mutant level and outcome (Whitman *et al*, 2008), although as noted above and in chapter 4, this study only included small numbers of FLT3/TKD⁺ patients with a markedly different distribution of mutant levels compared with the present analysis. It is also noteworthy that the clinical significance of mutant level is different for FLT3/ITDs and FLT3/TKDs in our cohort (Gale *et al*, 2007). Whilst low level FLT3/ITDs retained an adverse prognostic impact compared with FLT3/WT patients, low level FLT3/TKD cases could not be distinguished from FLT3/WT patients. Conversely, both high level FLT3/ITD⁺ and FLT3/TKD⁺ patients had highly significant and opposite impacts on CIR, DFS and OS.

As discussed above, mutant levels may reflect the stage at which the mutation was acquired, with lower level mutants occurring only as a secondary event in an already transformed cell, whereas higher level mutants are more likely to be earlier events in the transformation process. It is noteworthy, therefore, that the relatively favourable prognosis of young adult patients with FLT3/TKD⁺ AML is, in a large part, attributable to a reduction in the RR of mutant-positive patients who have entered CR. The opposite is the case for FLT3/ITDs. As relapse is thought to be caused by residual LSCs which are capable of repopulating the BM with a complete leukaemic hierarchy, stratification of patients

according to the mutant level may, therefore, correlate with presence of the mutation within the LSC population. Of relevance to this, it has been shown that FLT3/ITDs are present in the CD34+/CD38- population which is enriched for LSCs (Levis *et al*, 2005a).

Furthermore, another study used colony assays to demonstrate that the presence of FLT3/ITDs in this CD34+/CD38- population of cells is heterogeneous, and in some cases the FLT3/ITD is absent in this population of cells. Presence of a FLT3/ITD in this progenitor population correlated both with higher mutant level and also adverse outcome in paediatric AML patients (Pollard *et al*, 2006).

Studies of samples at presentation and relapse provided further biological insights into the differences between FLT3/ITDs and FLT3/TKDs. There were 9 FLT3/TKD⁺ patients where mutation status was available at relapse. Of 5 patients with a high level of mutation, 3 relapsed with the same mutation at a similar level. However, the other 2 high level mutants and all 4 patients with a lower level of mutation lost the mutation at relapse. This instability has been noted in an earlier study that showed FLT3/TKD mutations were lost at relapse in 8 of 13 (62%) cases studied (Shih *et al*, 2004). Furthermore, subsequent to the present analysis, another study also reported that of 13 FLT3/TKD⁺ cases at diagnosis, the mutation was lost at relapse in 9 (69%) of these (Bacher *et al*, 2008). This indicates that, although a FLT3/TKD mutation may contribute to the leukaemic phenotype, in many cases, it is not an essential requirement for the leukaemic clone (Figure 5.2). This supports the above evidence from the mutant level analysis that FLT3/TKDs are frequently late events in leukaemogenesis. In contrast, loss of FLT3/ITD mutations at relapse is less frequent than for FLT3/TKDs (Kottaridis *et al*, 2002, Schnittger *et al*, 2004, Shih *et al*, 2002). Furthermore, FLT3/ITD mutant level often increases at relapse whereas this is not the case for FLT3/TKDs (Shih *et al*, 2004), suggesting that FLT3/ITD⁺ cases may be more chemoresistant than FLT3/TKD⁺ cases.

The studies described in this chapter provide further insights into the biological differences between FLT3/ITDs and FLT3/TKDs. Distribution of mutant level complemented by presentation/relapse studies suggest that FLT3/TKDs are more frequently acquired as late or secondary events in leukaemogenesis than are FLT3/ITDs. This does not explain the differences observed in the clinical outcome associated with the two types of mutation, however, with high level FLT3/TKD mutations being associated with a particularly

favourable prognosis compared with the markedly adverse prognosis of high level FLT3/ITDs. Dissecting out the factors that determine these differences may provide valuable insights into the mechanisms of chemoresistance in AML.

CHAPTER 6: STUDIES OF THE CYTOTOXIC EFFECT OF FLT3 INHIBITORS AND CYTARABINE ON PRIMARY AML BLAST CELLS

6.1 Introduction

Studies to identify and characterise molecular defects in AML are partly driven by the goal of improving outcome for patients with this disease. The first way this may be achieved is through the identification of prognostic factors, thereby allowing the development of a more robust prognostic index in AML. It is hoped that this will, in turn, refine prognostic stratification of patients with AML to allow more accurate risk-adapted therapy. The second, and more direct potential benefit, is the identification of molecular targets for novel therapeutic approaches. FLT3 has emerged as an important candidate molecular marker for both these approaches. In view of the markedly increased RR associated with FLT3/ITDs, it is particularly important to determine whether treatment can be modified for this poor prognostic group of patients in order to improve their outcome. Interest in the optimal therapeutic strategy for patients with FLT3 mutations has mainly focused on two areas:

- Can the adverse outcome associated with FLT3/ITDs in AML be overcome using conventional therapeutic approaches?
- Do FLT3 inhibitors have therapeutic efficacy in patients with FLT3 mutated AML?

6.1.1 Can conventional therapeutic strategies be used to overcome the adverse prognosis associated with FLT3/ITDs?

There is no doubt that the combination of high dose chemoradiotherapy and graft-versus-leukaemia effect associated with allogeneic stem cell transplantation (SCT) dramatically reduces the RR in AML. However, this does not translate into a survival advantage in most studies because of the significant toxicity associated with the procedure (Burnett 2002b). Using conventional cytogenetic risk stratification, a recent study has suggested that patients in an intermediate or adverse cytogenetic risk group benefited from allogeneic SCT whereas those with favourable cytogenetics did not (Cornelissen *et al*, 2007). It is also

possible that FLT3/ITD⁺ patients would benefit from allogeneic SCT and should therefore be selected for transplant. The corollary of this would be that FLT3/ITD⁻ patients with an intermediate karyotype may not benefit from allogeneic SCT. This question was addressed in a study of 170 young adults with AML who received a Cy/TBI conditioned sibling allograft as part of the UK MRC AML 10 and 12 trials, 35 of whom were FLT3/ITD⁺ (Gale *et al*, 2005a). Allograft recipients had a 5 year RR which was comparable between FLT3/ITD⁺ (31%) and FLT3/ITD⁻ patients (25%) as opposed to 76% and 53% for those treated with chemotherapy alone. Although this suggests that the adverse RR associated with FLT3/ITDs was overcome by the allograft, because of the relatively small numbers of patients studied, the confidence intervals were wide and this apparent effect was not significant. The OS was 44% and 58% for FLT3/ITD⁺ and FLT3/WT patients respectively. Importantly, there was no evidence of any heterogeneity of effect of an allograft on relapse or survival when stratified according to FLT3/ITD status. Recent evidence suggests that the combination of NPM1 and FLT3/ITD mutation status may help to refine the process of patient selection for allogeneic transplantation (Schlenk *et al*, 2008), although these data will need to be validated in other studies.

Notwithstanding the lack of clear evidence, some centres are currently selecting FLT3/ITD mutant positive patients for allogeneic SCT. It has also been suggested that a similar strategy should apply to FLT3/TKD mutant positive patients as their prognostic impact was thought to be similar to that of FLT3/ITDs (Litzow 2005, Yanada *et al*, 2005). The data presented in chapters 4 and 5, however, suggest that this approach would be inappropriate in patients with FLT3/TKD mutations as the risks of the procedure may outweigh the benefits. It could even be proposed that patients with a high level FLT3/TKD mutation should be excluded from receiving an allogeneic transplant in view of their favourable prognosis. However, given the difficulty associated with providing statistically significant evidence for FLT3/ITDs (which are relatively frequent in AML), it would be extremely difficult to produce robust data to support this course of action for FLT3/TKDs. This is exemplified by the fact that in the total cohort of 1107 non-APL AML patients screened for FLT3/TKD mutations, only 17 of the 127 mutant-positive patients received an allograft. To demonstrate any survival advantage for high level FLT3/TKD mutant positive patients treated with chemotherapy alone compared to those who received an allograft in first CR would require a cohort of many thousands of patients.

6.1.2 Targeted therapies for human malignancies

Modification of conventional chemotherapeutic approaches with SCT is not the only approach for improving outcome for patients with leukaemia. The introduction of tyrosine kinase inhibitors has led to the development of many so called ‘targeted therapies’ against oncogenic tyrosine kinases (Krause and Van Etten 2005). The remarkable success of imatinib in the treatment of chronic myeloid leukaemia (CML) (Druker *et al*, 2001) has served as a paradigm for this approach. CML is characterised by the t(9;22) Philadelphia chromosome which leads to the production of a bcr-abl fusion protein, with consequent constitutive activation of the abl tyrosine kinase. Imatinib is an abl kinase inhibitor which was shown to be cytotoxic to bcr-abl expressing cell lines and primary CML leukaemic cells *in vitro* (Druker *et al*, 1996). Initial clinical trials of imatinib were in chronic phase CML patients who had failed therapy with interferon- α . Single agent therapy with imatinib was remarkably effective and induced a complete haematological remission in 53 of 54 patients when used at doses of 300mg per day or more. Furthermore, major cytogenetic responses occurred in 17 of 54 patients in this early study (Druker *et al*, 2001). Larger randomised studies subsequently demonstrated superiority of imatinib for the first line therapy of chronic phase CML compared to interferon- α with or without cytarabine. The complete cytogenetic response rate at 18 months in the two treatment groups was 76.2% and 14.5% respectively (O'Brien *et al*, 2003). This has led to a significant reduction in the number of patients receiving an allogeneic transplant in this disorder (Giralt *et al*, 2007).

6.1.3 FLT3 inhibitors

In view of the success of imatinib, there has been a great deal of interest in the development of other small molecule inhibitors to target dysregulated tyrosine kinases in other malignancies (Krause and Van Etten 2005). There are a number of reasons why FLT3 is an attractive therapeutic target for such an approach. Firstly, constitutive activation of FLT3 is a frequent occurrence in AML due to the approximately one quarter of young adults with AML that have ITDs within the juxtamembrane domain, and the 7-22% of patients with mutations within the TKD (See chapter 3). Furthermore, activation of WT FLT3 may also occur as a result of autocrine stimulation by FLT3-ligand (Zheng *et al*, 2004b), over-expression of FLT3 (Armstrong *et al*, 2003, Dicker *et al*, 2007) or by inactivating mutations of c-cbl (Caligiuri *et al*, 2007, Sargin *et al*, 2007). Targeting FLT3 in AML has

generated particular enthusiasm in view of the poor prognosis associated with FLT3/ITDs (Kottaridis *et al*, 2001).

The first inhibitor of FLT3 function to be described was herbimycin A. Rather than directly inhibiting the FLT3 kinase, herbimycin A inhibits the molecular chaperone Hsp-90, thereby interfering with FLT3/ITD protein folding. This agent was shown to inhibit the growth of transformed 32Dcl3 cells (Zhao *et al*, 2000), an effect that intriguingly appears to be specific to FLT3/ITDs and not FLT3/WT (Minami *et al*, 2002, Zhao *et al*, 2000). The first direct inhibitors of the FLT3 tyrosine kinase to be discovered were the bicyclic quinoxalines AG1295 (Levis *et al*, 2001) and AG1296 (Tse *et al*, 2001). Both were initially described as inhibitors of PDGFR β , a closely related class 3 RTK. These agents compete with ATP for the ATP binding pocket, and inhibit the FLT3 kinase with an IC₅₀ of approximately 300 nM. AG1296 was shown to inhibit the factor independent proliferation of Ba/F3 cells (Tse *et al*, 2001) and AG1295 was shown to be specifically cytotoxic against FLT3/ITD⁺ AML blast cells *in vitro* (Levis *et al*, 2001). These inhibitors however have a very low bioavailability due to their hydrophobicity and therefore have little potential as clinical agents.

Drug discovery screening programmes in the pharmaceutical industry have now identified a wide array of different small molecule inhibitors of FLT3 from a number of different chemical classes (Knapper 2007) and with variable specificity for the FLT3 kinase (Table 6.1). Many of these inhibitors are still at the preclinical stage, however, a number of these agents have progressed to more advanced stages of clinical development and these are summarised in Table 6.2. Inevitably, these phase 1/2 studies have been primarily conducted in patients with relapsed or refractory disease. Importantly, the agents have generally been well tolerated at doses which achieved serum concentrations of the drug sufficient to inhibit FLT3 phosphorylation *in vitro*. Despite the high-risk patients enrolled, clinical responses have been observed and are generally characterised by reductions in the WBC, with some cases clearing the BM to <5% blasts. These responses have occurred in both FLT3 mutant and FLT3/WT patients. However, it should be noted that, at the present time, no CRs have been induced using a FLT3 inhibitor as monotherapy. Furthermore, the clinical responses that have been seen have been transient, with progression of the disease within weeks.

Table 6.1: Inhibitors of the FLT3 tyrosine kinase

Name	Chemical Class	IC50 FLT3	Other kinases	Stage of Development	Reference
ABT-869	Urea derivative	4nM	KIT, KDR, PDGFR	Phase I	(Zhou <i>et al</i> , 2008)
AG1295	Quinoxaline	300nM	PDGFR, KIT	Preclinical	(Levis <i>et al</i> , 2001)
AG1296	Quinoxaline	300 nM	PDGFR, KIT	Preclinical	(Tse <i>et al</i> , 2001)
AGL2043	Quinoxaline	100 nM	PDGFR, KIT	Preclinical	(Gazit <i>et al</i> , 2003)
AS602868	Anilinopyrimidine	678 nM	IKK2	Preclinical	(Griessinger <i>et al</i> , 2007)
BAY 43-9006 (Sorafenib)	Bi-aryl urea	<50nM	B-RAF, VEGFR, PDGFR	Preclinical	(Zhang <i>et al</i> , 2008)
CEP701 (lestaurtinib)	Indolocarbazole	2 nM	TRKA, VEGFR	Phase III	(Levis <i>et al</i> , 2002)
CHIR-258 (TKI258)	Amino-benzimidazole-quinolinone	10 nM	KIT, FMS, FGFR, VEGFR, PDGFR	Phase I	(Trudel <i>et al</i> , 2005)
D64406	Bis-indolyl-1-methanone	300 nM	PDGFR, KIT	Preclinical	(Teller <i>et al</i> , 2002)
FI-700	Not reported	20 nM	KIT, FMS, LIN	Preclinical	(Kiyoi <i>et al</i> , 2007)
Go6976	Indolocarbazole		JAK2, PKC	Preclinical	(Grandage <i>et al</i> , 2006)
GTP-14564	Cyclopenta[a]inden	300 nM	KIT, FMS, PDGFR	Preclinical	(Murata <i>et al</i> , 2003)
Herbimycin A	Benzoquinoid ansamycin	100 nM	PDGFR, EGFR	Preclinical	(Zhao <i>et al</i> , 2000)
IMC-EB10	Monoclonal Antibody	N/A	NR	Preclinical	(Piloto <i>et al</i> , 2005)
IMC-NC7	Monoclonal Antibody	N/A	NR	Preclinical	(Piloto <i>et al</i> , 2005)
Ki23819	Quinoline-urea	10 nM	NR	Preclinical	(Komeno <i>et al</i> , 2005)
KRN383	Quinoline-urea	3 nM	NR	Preclinical	(Nishiyama <i>et al</i> , 2006)
KW-2449	NR	6 nM	KIT, Aurora	Phase I	(Pratz <i>et al</i> , 2009)
LS-104	Hydroxystyryl–acrylonitrile compound	4 µM	ABL, JAK2	Phase I	(Kasper <i>et al</i> , 2008)
MLN518	Piperazinyl quinazoline	30 nM	KIT, PDGFR	Phase II	(Kelly <i>et al</i> , 2002c)
NVP-AST487	N,N'-diphenyl urea	120 nM	RET, c-Kit, ABL	Preclinical	(Weisberg <i>et al</i> , 2008)
PKC412	Indolocarbazole	10 nM	KIT, KDR, PKC, PDGFR	Phase III	(Weisberg <i>et al</i> , 2002)
SU5416	3-Substituted indolinone	100 nM	KIT, VEGFR	Phase II	(Yee <i>et al</i> , 2002)
SU5614	3-Substituted indolinone	10 nM	KIT, FMS	Preclinical	(Yee <i>et al</i> , 2002)
SU11248 (sunitinib)	3-Substituted indolinoneindolinone	50 nM	KIT, PDGFR, VEGFR	Phase I	(O'Farrell <i>et al</i> , 2003)

Table 6.2: Clinical trials of FLT3 inhibitors in patients with AML

Trial	Agent	No. FLT3 WT/ITD/TKD	Median Age (range)	Disease Status	Significant clinical benefit	CR	Time to Progression in responders
(Giles <i>et al</i>, 2003)	SU5416	33 (unknown mutation status)	64 (23 – 76)	U + R + Rf	3/33 (1 TKD +ve)	0	Event free survival 3-6.5 months
(O'Farrell <i>et al</i>, 2003)	SU11248	24/3/2	67 (19-82)	U + R + Rf	5/29 (2 ITDs, 3 WT)	0	NA
(Fiedler <i>et al</i>, 2003)	SU5416	28/7/NA 7 unknown	65 (27-79)	U + R + Rf	8/42 (0/7 FLT3/ITD)	0	Median 1.6 months
(Smith <i>et al</i>, 2004)	Lestaurtinib (CEP701)	0/14/0	61.5 (18-74)	R + Rf	5/14	0	2 weeks to 3 months
(Fiedler <i>et al</i>, 2005)	SU11248	10/2/2	70.5 (54-80)	U + Rf	6/14 (4/4 mutant FLT3)	0	Median 2 cycles (6 weeks per cycle)
(Stone <i>et al</i>, 2005b)	PKC412	0/18/2	62 (29-78)	R + Rf	14/20	0	Median 13 weeks
(De Angelo <i>et al</i>, 2006)	Tandutinib (MLN518)	30/8/1	70.5	U + R + Rf	2/39 (2/8 FLT3/ITDs)	0	44 days and 2 months
(Knapper <i>et al</i>, 2006a)	Lestaurtinib (CEP701)	22/2/3	73 (67 – 82)	U	8/27 (3/5 mutant FLT3)	0	Median 28 days

U indicates untreated patients not fit for conventional chemotherapy, R relapsed patients, Rf refractory patients, NA not available.

Two indolocarbazole compounds, lestaurtinib and PKC412, originally derived from the alkaloid K-252a (a fermentation product of *Nonomurea longicatena*) are competitive inhibitors of the FLT3 kinase and are of particular interest as they have both shown some efficacy as single agents in AML. Both have progressed to ongoing multi-centre phase 3 trials in AML. Lestaurtinib, originally known as CEP701, is a potent and relatively specific inhibitor of the FLT3 kinase with an IC₅₀ of approximately 2nM (Levis *et al*, 2002), as opposed to IC₅₀s of over 500 nM for other class 3 RTKs. It was originally identified as an inhibitor of TrkA (IC₅₀ 3 nM), a member of the nerve growth factor receptor subfamily (non-class III RTK). It has been shown to have a cytotoxic effect against AML blasts *in vitro*, particularly cases which are FLT3/ITD⁺ compared to FLT3/WT cases, and has also been shown to prolong survival in a mouse model of FLT3/ITDs (Brown *et al*, 2004, Knapper *et al*, 2006b, Levis *et al*, 2002). In clinical trials, lestaurtinib has good oral bioavailability and is well tolerated, with gastrointestinal side effects being the most common toxicity. One phase 1/2 study assessed the efficacy of CEP701 in 14 relapsed or refractory FLT3/ITD⁺ AML patients. Transient responses were seen in 5 patients, including one patient in whom the BM blast percentage reduced to <5% (Smith *et al*, 2004). A second phase 2 trial of lestaurtinib in elderly patients with untreated AML demonstrated transient responses in 3 of 5 patients with a FLT3 mutation and 5 of 22 patients with FLT3/WT (Knapper *et al*, 2006a). Importantly, both of these trials demonstrated that a patient was highly likely to demonstrate a clinical response if their blast cells were sensitive to lestaurtinib inhibition *in vitro* and if plasma levels of lestaurtinib sufficient for *in vitro* FLT3 inhibition were achieved.

PKC412 is another orally bioavailable indolocarbazole which inhibits FLT3 with an IC₅₀ of approximately 10nM. This agent was originally described as an inhibitor of protein kinase C and is less selective against FLT3 than lestaurtinib, with significant inhibitory activity against PDGFR β and c-Kit. It was shown to inhibit the growth and cause apoptosis of FLT3 transformed Ba/F3 cells, and prolong survival in mice transplanted with BM cells oncoretrovirally transduced with a FLT3/ITD (Weisberg *et al*, 2002). In a phase 2 study of 20 patients with FLT3 mutated relapsed/refractory AML, 14 patients demonstrated a significant reduction in WBC, with 2 cases clearing BM blasts to <5%. Again, responses were transient, with a median duration of 13 weeks (Stone *et al*, 2005b). Interestingly,

much of the clinical activity of PKC412 appears to relate to a metabolite (CGP52541) rather than PKC412 itself (Levis *et al*, 2006).

Despite the lack of durable remissions, the demonstration of a biological effect of FLT3 inhibition in preclinical and clinical studies of FLT3/ITD mutants has been sufficiently encouraging for these agents to be taken forward into larger clinical trials. Since several studies have shown that FLT3/ITD⁺ AML blast cells are more sensitive to FLT3 inhibitors than FLT3/WT cases, both *in vitro* and *in vivo* (Brown *et al*, 2004, Knapper 2007, Knapper *et al*, 2006b, Levis *et al*, 2002), a number of these trials only include FLT3 mutant-positive cases, defined as either FLT3/ITD⁺ or FLT3/TKD⁺. As demonstrated in chapters 3 and 4 however, in the cohort studied here, FLT3/TKD mutations are biologically distinct from FLT3/ITDs and are an independent predictor of favourable outcome in AML. Moreover, as described in chapter 5, whilst high levels of a FLT3/ITD mutation are associated with a particularly adverse prognosis (Gale *et al*, 2007, Thiede *et al*, 2002, Whitman *et al*, 2001), the opposite is the case for FLT3/TKDs. These data raise the possibility that the *in vitro* and *in vivo* effects of FLT3 inhibitors will also differ according to the mechanism of FLT3 activation. Whether FLT3/TKD⁺ cases are more sensitive to FLT3 inhibition than FLT3/WT cases, however, is unclear. This question is important as some clinical trials of FLT3 inhibitors include FLT3/TKD⁺ patients but exclude FLT3/WT cases. The only study to address this issue in adult AML examined the *in vitro* effects of lestaurtinib on FLT3/TKD⁺ cases and suggested that they respond similarly to FLT3/ITD⁺ cases (Knapper *et al*, 2006b), although only 6 FLT3/TKD⁺ cases were studied. A study in paediatric AML, however, suggested that FLT3/ITD⁺ and FLT3/TKD⁺ cases differ in their response to lestaurtinib (Brown *et al*, 2004).

To determine whether the clinical and biological differences between FLT3/ITDs and FLT3/TKDs influence the response to FLT3 inhibitors, the *in vitro* effects of lestaurtinib and PKC412 on primary AML blast cells, both alone and in combination with cytarabine, were studied. Results were correlated with FLT3 mutation status and mutant level.

6.2 Materials and Methods

6.2.1 Patients

Patient details are shown in Table 6.3. Mononuclear cells from the peripheral blood or bone marrow of patients with newly diagnosed (n=31) or relapsed (n=5) AML were purified using Ficoll-Hypaque centrifugation (Chapter 2.1.7). Where possible, cells were used fresh (n=22), otherwise they were stored in liquid nitrogen until required (n=14). Cryopreserved cells were thawed (Chapter 2.1.8) and live cells were purified using Ficoll-Hypaque centrifugation. At the time of analysis, all samples had 90% or more viable cells as determined by trypan blue exclusion (Chapter 2.1.9). Informed consent for all tissue collection was provided according to the Declaration of Helsinki.

6.2.2 Reagents

Lestaurtinib and PKC412 were stored as stock solutions at concentrations of 4mM in DMSO at -20°C. A working solution of 100µM was made up prior to each experiment by dilution in RPMI. Cytarabine was stored as a 100mg/ml stock solution at 4°C.

6.2.3 Detection and Identification of FLT3 Mutations

FLT3/TKD mutations were detected, identified and quantified as previously described in chapters 3 and 4. FLT3/ITD status and mutant level were known from previous work in the department (Gale *et al*, 2005a).

6.2.4 MTS Assays

Purified cells were resuspended in 96 well plates at 2×10^5 /100µl in RPMI containing 10% fetal calf serum (R10) in the presence or absence of cytarabine (100, 10, 1 or 0.1µg/ml), lestaurtinib or PKC412 (100, 50, 20, 10 or 5nM) or, when enough cells were available, at least six different combinations of lestaurtinib and cytarabine. Each condition was plated in triplicate. Cells were incubated at 37°C, 5% CO₂ in a humidified incubator. After 48 hours, 20 µl of MTS tetrazolium compound (Promega, Southampton, UK) was added to each well and absorbance read at 490nm after 4 hours. The effects of cytarabine and lestaurtinib as single agents were expressed as a percentage of optical density (OD) relative to untreated cells (\pm standard error of the mean [SEM]).

6.2.5 Sequential incubation with lestaurtinib and cytarabine

In 10 cases where adequate numbers of cells were available, 4×10^6 cells were incubated in R10 at 2×10^6 /ml in the presence of either nothing, 1 μ g/ml cytarabine, 20nM lestaurtinib or a combination of the two. After 48 hours the cells from each of the 4 conditions were then washed twice in R10, split into 4 aliquots and re-suspended in each of the same 4 conditions without adjustment for cell number, making a total of 16 different conditions. The cells were incubated for a further 48 hours; cell viability was then assessed using the MTS assay as above. The results were expressed as the optical density relative to cells cultured in R10 only for 96 hours.

6.2.6 Data Analysis and Statistical Interpretation

P-values are for the student T-test between two groups. The relationship between mutant level and cytotoxicity was calculated using Spearman's Rank Correlation. The median dose effect (Dm) was calculated using Calcsyn software. Evidence of synergy between cytarabine and lestaurtinib was assessed using the combination index (CI) of Chou and Talalay (Chou and Talalay 1984) (Calcsyn software). A CI of less than 1 indicates evidence of a synergistic interaction.

6.3 Results

6.3.1 Comparison of the cytotoxic effect of PKC412 and lestaurtinib on primary AML blast cells

The cytotoxic effect of both PKC412 and lestaurtinib was tested on a small cohort of blast cells from 7 patients with AML. Of these 7 patients, 3 were FLT3/WT, 3 were FLT3/ITD⁺ and 1 was FLT3/TKD⁺. The median age was 44 years (range 21 – 61). The *in vitro* cytotoxic effect of lestaurtinib was greater than that of PKC412 at all concentrations tested (Figure 6.1). For example, at a concentration of 50nM, the mean %OD for lestaurtinib was $63.9 \pm 8.5\%$ (SEM) compared to $88.2 \pm 6.8\%$ for PKC412 ($P=0.05$). In view of the lack of *in vitro* dose response for PKC412, subsequent experiments were performed with lestaurtinib only.

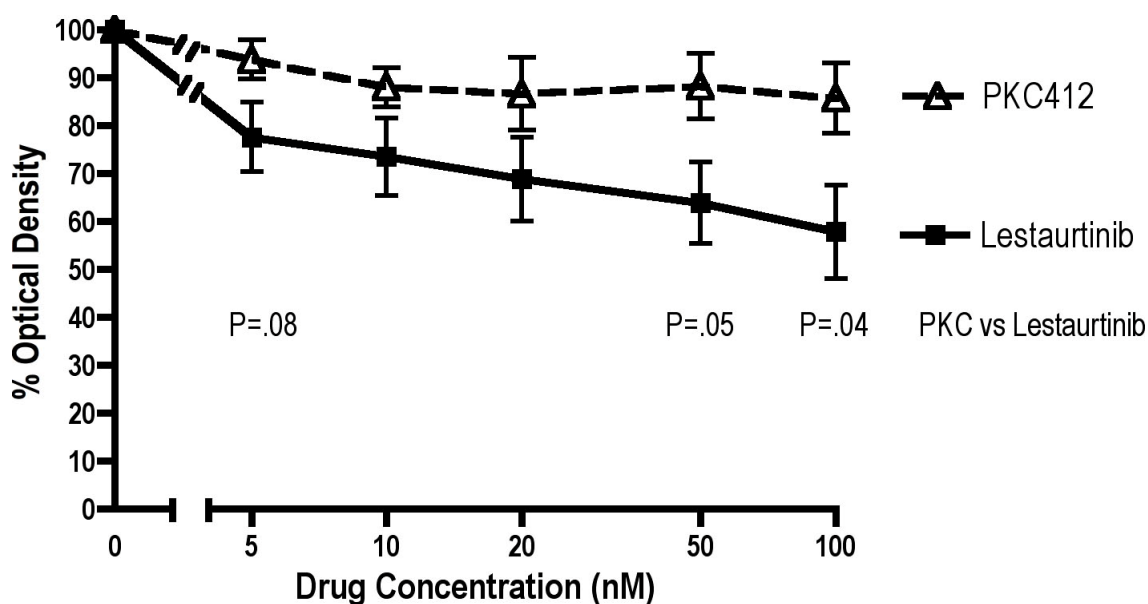


Figure 6.1: Cytotoxic dose-response to lestaurtinib and PKC412 of blast cells from 7 patients with AML. Error bars represent standard error of the mean.

6.3.2 Characteristic of the whole cohort of patients studied

Of the 36 cases examined for the cytotoxic effect of lestaurtinib, 14 were FLT3/WT, 11 were FLT3/ITD⁺ with a median mutant level of 45% of total FLT3 alleles (range 17%-93%), and 11 were FLT3/TKD⁺ (Table 6.3). The mutation was identified in 10 FLT3/TKD⁺ cases, 3 were D835Y, 3 Δ836, and a single case each of D835V, N841I, D839G and a 12 base pair insertion, with mutant levels varying between 22% and 50% of total FLT3 alleles. The one uncharacterised FLT3/TKD mutation was present at a low level, approximately 5% of FLT3 alleles.

6.3.3 The cytotoxic effect of cytarabine on primary AML blast cells with different FLT3 mutation status

Overall, there was no difference in the cytotoxic effect of cytarabine between FLT3/ITD⁺, FLT3/TKD⁺ and FLT3/WT blast cells (Figure 6.2A). At a concentration of 10 μg/ml cytarabine, the mean %OD for these three groups was 45±5%, 43±7% and 50±6% respectively (Figure 6.2B). However, in FLT3/ITD⁺ cases, there was a moderate correlation between increasing mutant level and an increased resistance to cytarabine-induced cytotoxicity ($r^2=0.48$, $P=.04$) which was not apparent in FLT3/TKD⁺ cases (Figure 6.2D).

Table 6.3: Clinical and demographic characteristics of patients stratified by FLT3 mutation status.

	FLT3/WT	FLT3/ITD+ve	FLT3/TKD+ve
Number	14	11	11
Sex (M/F)	12/2	8/3	3/8
Median Age in years (Range)	49 (17-77)	56 (21-67)	44 (24-73)
Disease Stage			
Presentation	12	9	10
Relapse	2	2	1
Cytogenetics			
Favorable	5	1	2
Intermediate	6	8	8
Adverse	2	0	0
Unknown	1	2	1
FLT3 Mutation			
Median Mutant Level (%) (Range)	N/A	45 (17-93)	43 (5-50)

6.3.4 The cytotoxic effect of lestaurtinib on primary AML blast cells with different FLT3 mutation status

Lestaurtinib induced increased cytotoxicity in FLT3/ITD⁺ compared to FLT3/WT blasts (Figure 6.3A). FLT3/TKD⁺ blast cells, however, responded similarly to FLT3/WT cells at all concentrations tested. At 5nM lestaurtinib, there was a highly significant difference between FLT3/ITD⁺ cells compared to FLT3/TKD⁺ (P=.006) and FLT3/WT (P=.004) cells, with a mean %OD of 70±6%, 92±2% and 93±3% respectively (Figure 6.3B). There was no correlation between FLT3/ITD or FLT3/TKD mutant level and sensitivity to lestaurtinib (Figures 6.3C and 6.3D). It is noteworthy, however, that the two very high level FLT3/ITD⁺ cases (83% and 93% mutant) were quite sensitive to lestaurtinib (%OD at 5nM lestaurtinib, 54% and 65% respectively compared to the median of 70%) but were relatively resistant to cytarabine (%OD at 10µg/ml cytarabine, 60% and 62% respectively compared to the median of 45%) (Figures 6.2C and 6.3C).

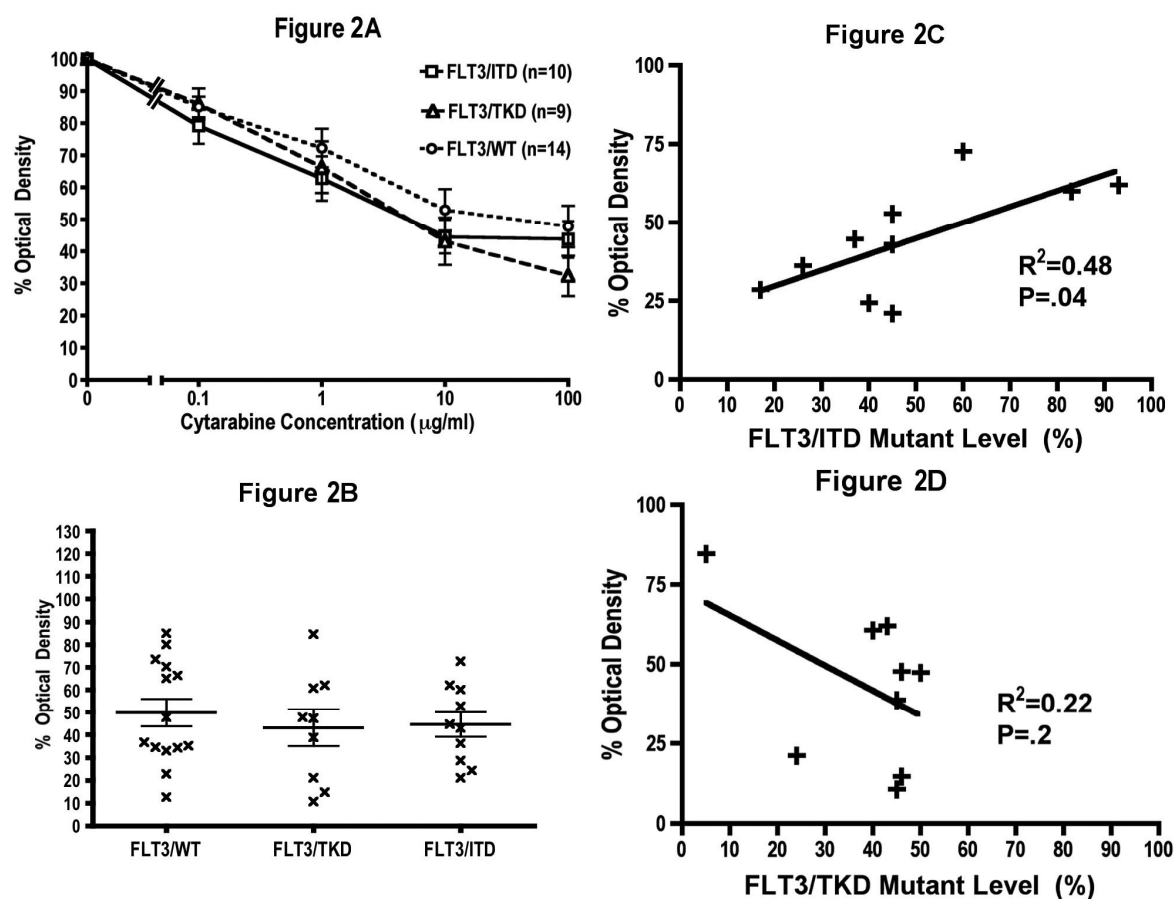


Figure 6.2: Cytotoxic responses to cytarabine stratified according to FLT3 mutation status. (A) Dose response to cytarabine, (B) Scatter plot of individual responses to 10 µg/ml cytarabine, (C) Relationship between response to 10 µg/ml cytarabine and FLT3/ITD mutant level, (D) Relationship between response to 10 µg/ml cytarabine and FLT3/TKD mutant level. Error bars represent standard error of the mean.

6.3.5 The cytotoxic effect of a combination of cytarabine and lestaurtinib on primary AML blast cells with different FLT3 mutation status

The cytotoxic effect of combinations of lestaurtinib and cytarabine was studied in 33 cases, 14 FLT3/WT, 9 FLT3/TKD⁺ and 10 FLT3/ITD⁺. The cytotoxic effect of lestaurtinib was synergistic with that of cytarabine at the majority of combinations used. The median CI (with 25th-75th percentiles) was 0.47 (0.27 – 1.1) for FLT3/WT cells, 0.59 (0.23 – 1.49) for FLT3/TKD⁺ cells and 0.35 (0.24 – 0.7) for FLT3/ITD⁺ cells respectively (Figure 6.4),

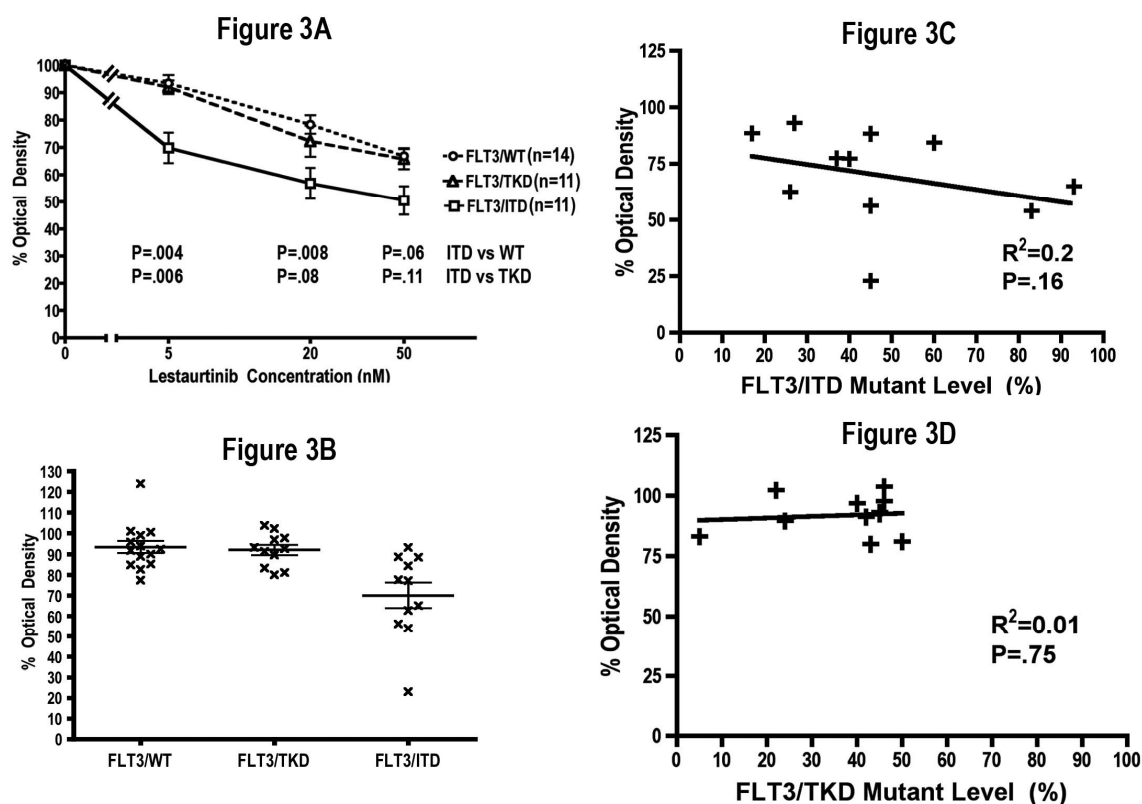


Figure 6.3: Cytotoxic responses to lestaurtinib stratified according to FLT3 mutation status. (A) Dose response to lestaurtinib, (B) Scatter plot of individual responses to 5nM lestaurtinib, (C) Relationship between response to 5nM lestaurtinib and FLT3/ITD mutant level, (D) Relationship between response to 5nM lestaurtinib and FLT3/TKD mutant level. Error bars represent standard error of the mean. indicating that the highest degree of synergy was in FLT3/ITD⁺ samples, although the differences between the three groups were not statistically significant.

6.3.6 Sequential incubation of primary AML cells with cytarabine and lestaurtinib

The impact of sequential administration of cytarabine and lestaurtinib on their cytotoxic effect was studied in 10 cases. The results were adequate for interpretation in 6 of the 10 cases studied. In the other 4 cases the MTS colour change of the control cells was inadequate for analysis (<0.2 mean optical density). Of the 6 cases studied, 2 were FLT3/WT, 2 were FLT3/ITD⁺ and 2 were FLT3/TKD⁺. The results of the sequential administration experiments are shown in Figure 6.5 and Table 6.4. Blast cells treated initially with lestaurtinib then cytarabine had a mean %OD of 56±9% versus 46±7% for

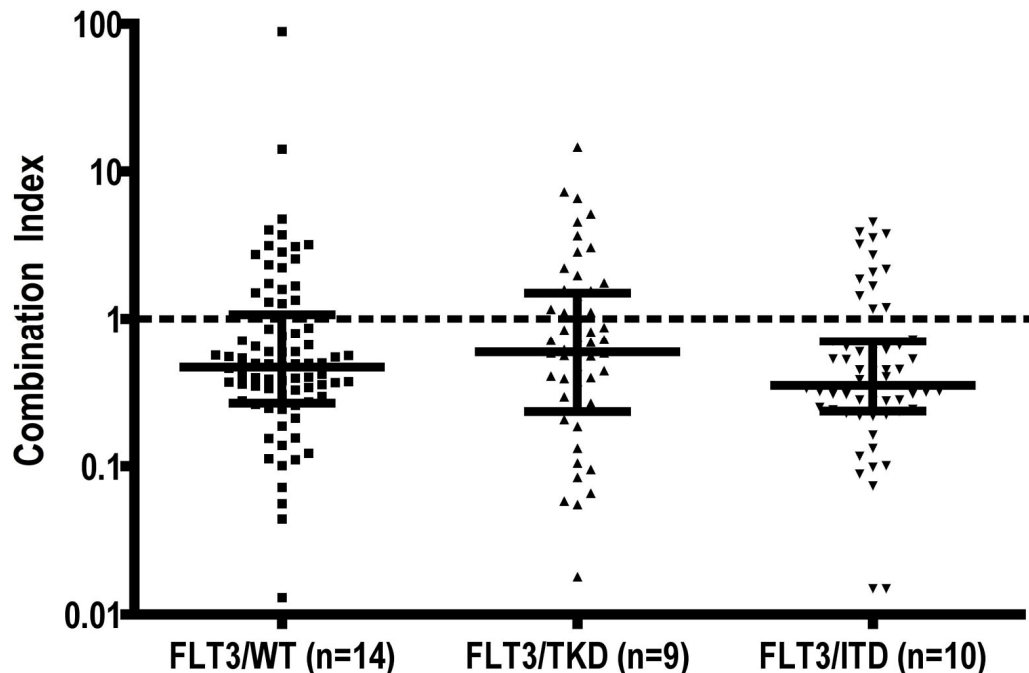


Figure 6.4: Scatter plot of the cytotoxic effect of lestaurtinib in combination with cytarabine. The combination index (CI) for the six different combinations of the two drugs is plotted for each sample and stratified according to FLT3 mutation status. A CI of 1, shown by the dashed line, is consistent with an additive effect when the two agents are combined. A CI of less than 1 indicates evidence of a synergistic interaction and a CI of greater than 1 indicates an antagonistic interaction. Error bars represent the median and 25th - 75th percentiles for each group.

cells treated in the opposite sequence. The mean ratio of %OD for blast cells treated initially with cytarabine then lestaurtinib versus the opposite sequence was 0.82 (i.e. 46/56), representing an increased cell kill for cells cultured in cytarabine first. This may suggest that lestaurtinib is having an inhibitory effect on the cytotoxic effect of cytarabine. However, blast cells cultured in cytarabine followed by R10 had a %OD of $57 \pm 10\%$ versus $74 \pm 9\%$ for those treated in the opposite sequence with a ratio of 0.77. This demonstrates that the cell kill was always greater when cells were cultured in cytarabine for the first 48 hours compared to the second 48 hours, irrespective of the presence of lestaurtinib. This effect was not apparent with lestaurtinib; following incubation with lestaurtinib alone for the first 48 hours and then R10 for the second 48 hours the %OD was $74 \pm 9\%$ versus

77±6% for blast cells incubated in the opposite sequence (ratio 0.96). The greatest cytotoxic effect was achieved by continuous incubation with a combination of cytarabine and lestaurtinib for the whole 96 hours.

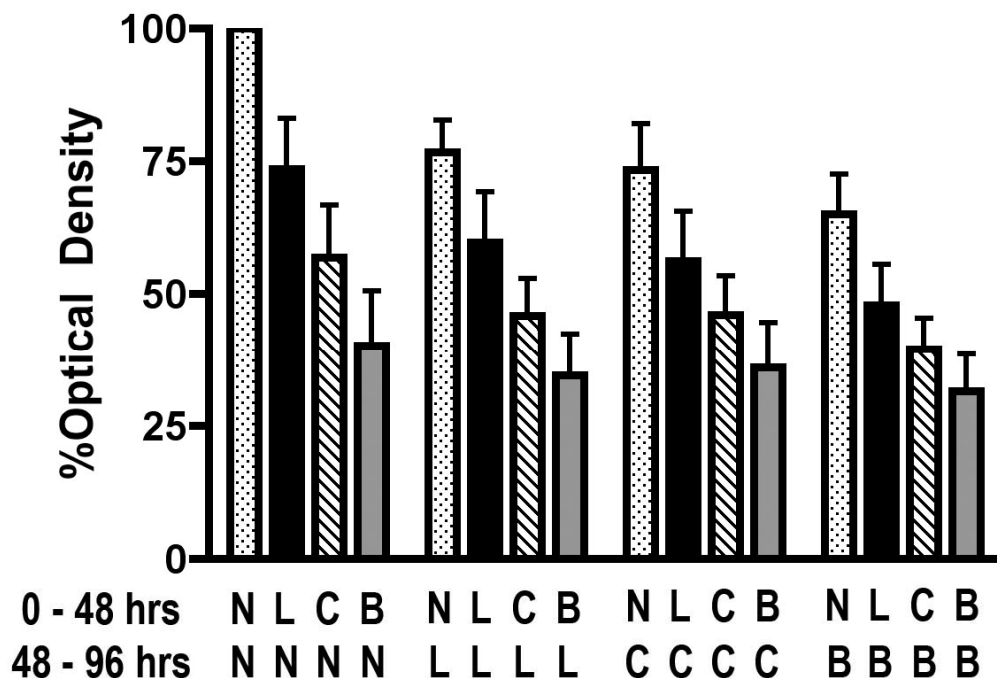


Figure 6.5: The impact of different sequences of administration of lestaurtinib on blast cells from 6 patients with AML. N indicates incubation in R10 only; L, 20 nM lestaurtinib only; C, 1 µg/ml cytarabine only; and B, both 1 µg/ml cytarabine and 20 nM lestaurtinib. Error bars represent the SEM.

Table 6.4: Sequential culture of blast cells in cytarabine and lestaurtinib. The percentage optical density is shown for each of the 16 different culture conditions after 96 hours of incubation.

Second 48 Hours	First 48 Hours			
	Nil	Lestaurtinib	Cytarabine	Both
Nil	100±0	74±9	57±10	40±10
Lestaurtinib	77±6	60±9	46±7	35±8
Cytarabine	74±9	56±9	46±7	36±8
Both	65±7	48±8	40±6	32±7

6.4 Discussion

As small molecule inhibitors that target FLT3 progress to more advanced clinical trials, it is becoming increasingly important to determine which patients might benefit from receiving them as part of their therapy. *In vitro* studies have shown that FLT3/ITD⁺ blast cells are more sensitive to lestaurtinib-induced cytotoxicity than FLT3/WT cases (Brown *et al*, 2004, Knapper *et al*, 2006b, Levis *et al*, 2002), however, although both types of mutation lead to constitutively activated receptors, the situation in FLT3/TKD⁺ blast cells is less clear. Relatively few samples from such cases have been studied due to the low frequency of these mutations, and whilst one study reported the same response to lestaurtinib as FLT3/ITD⁺ blasts, in another study they showed a reduced response, similar to FLT3/WT blasts (Brown *et al*, 2004, Knapper *et al*, 2006b). This is an important issue to clarify as an *in vitro* cytotoxic response closely correlates with clinical response in patients where adequate serum concentrations of the drug are achieved (Knapper *et al*, 2006a, Smith *et al*, 2004). The studies presented within this chapter therefore focussed on FLT3/TKD⁺ cases and found that the response to lestaurtinib of blast cells from 11 such cases was significantly less than that of FLT3/ITD⁺ cells but similar to FLT3/WT cells at all concentrations tested.

There are a number of possible explanations for the differential response of ITD and TKD mutations to FLT3 inhibition. It is possible that the impact of the mutation on protein conformation influences interaction with lestaurtinib. In FLT3/ITDs, the insertion of additional amino acids is thought to disrupt the juxtamembrane domain that normally stabilises all the structural components involved in holding the kinase in an inactive conformation (Gilliland and Griffin 2002a, Griffith *et al*, 2004). In FLT3/TKD mutations, however, the amino acid substitutions or deletions directly disturb the activation loop and the conformational change is more comparable to that induced by ligand stimulation of the WT receptor. FLT3/ITDs may, therefore, have a more powerful effect on protein unfolding than FLT3/TKD mutations and consequently lead to greater accessibility to lestaurtinib. However, in a cell line model, it has previously been reported that the two types of mutation have similar IC₅₀s for lestaurtinib (Levis *et al*, 2002), suggesting that this is not the reason for the difference. Furthermore, point mutations leading to resistance to FLT3

inhibition by indolocarbazoles occur in the ATP binding pocket in the first kinase domain and not the second kinase domain (Cools *et al*, 2004, Heidel *et al*, 2006).

An alternative explanation for the differential sensitivity of FLT3/ITD⁺ and FLT3/TKD⁺ blast cells to FLT3 inhibition may be that FLT3/ITD⁺ blast cells are more dependent on FLT3 signalling for survival than FLT3/TKD⁺ cells and are therefore more likely to die when FLT3 is inhibited. This may relate to differential events that occur downstream of the two types of mutation, for example, studies have indicated that, unlike FLT3/ITDs, FLT3/TKD mutations lead to signalling events that are very similar to those caused by ligand activation of the WT receptor (Choudhary *et al*, 2005b, Grundler *et al*, 2005). It should be noted, however, that response to lestaurtinib was not totally absent in either FLT3/TKD⁺ or FLT3/WT cases, particularly at the higher drug concentrations used, just less than that observed in FLT3/ITD⁺ cases. This suggests that, in at least a proportion of these cases, FLT3 signalling is an important contributor to leukaemia cell survival.

The level of a FLT3 mutation has been shown to add important prognostic information to the detection of the presence of a mutation alone, and higher mutant levels correlate with a significantly poorer outcome in FLT3/ITD⁺ cases (Gale *et al*, 2007, Meshinchi *et al*, 2006, Thiede *et al*, 2002, Whitman *et al*, 2001), and an improved outcome in FLT3/TKD⁺ cases (Mead *et al*, 2007). The relationship between mutant level and response to cytarabine and lestaurtinib was therefore examined. Previous studies have suggested that higher level FLT3/ITDs may be more sensitive to the effects of FLT3 inhibitors than lower level mutants (Brown *et al*, 2004, Knapper *et al*, 2006b). The data presented in this chapter demonstrated that there was a weak correlation between increasing lestaurtinib sensitivity and higher FLT3/ITD mutant level, but this was not statistically significant. However, there was evidence of a significant inverse relationship between cytarabine-induced cytotoxicity and FLT3/ITD mutant level. In particular, two cases had a FLT3/ITD mutant level that was consistent with loss of the FLT3/WT allele through mitotic recombination (Raghavan *et al*, 2005). It is noteworthy that these cases showed relative resistance to cytarabine but remained highly sensitive to lestaurtinib-induced cytotoxicity. This has important implications for treatment of such cases as they have a particularly adverse prognosis, with a very high relapse rate, and are good candidates for more experimental therapies. Furthermore, studies of paired presentation/relapse samples have shown that in FLT3/ITD⁺

cases, mutant levels are often increased at relapse, probably because the ITD-carrying leukaemic stem cells have increased chemo-resistance and are therefore selected for (Kottaridis *et al*, 2002, Schnittger *et al*, 2004, Shih *et al*, 2002). Sensitivity to targeted FLT3 inhibition may therefore be especially beneficial in such cases. There was no evidence of any relationship between FLT3/TKD mutant level and sensitivity to either cytarabine or lestaurtinib.

The modest clinical responses seen in clinical trials of FLT3 inhibitors (Table 6.2) may, at least in part, reflect the poor risk patients entered into these trials. Nevertheless, significant responses were seen, and given the molecular heterogeneity of AML in comparison to chronic phase CML, expectations for the efficacy of single agent FLT3 inhibitors need to be adjusted accordingly. Unfortunately however, all trials using FLT3 inhibitors as monotherapy have shown that resistance rapidly occurs in responding patients (Table 6.2). This may relate to new mutations in the kinase domain (Heidel *et al*, 2006), or the activation of downstream signalling by other mechanisms, thereby bypassing FLT3 (Piloto *et al*, 2006). Furthermore, in some cases, a FLT3 mutation is probably a secondary event occurring late in leukaemogenesis, with the FLT3 mutant(s) only being present in a subpopulation of leukaemic cells (Kottaridis *et al*, 2002). It is possible that in these cases there will be a rapid outgrowth of FLT3/WT leukaemic clones, resistant to the effects of FLT3 inhibition. Finally, the main cause of treatment failure in AML is relapse caused by residual leukaemic stem cells which survive induction and consolidation chemotherapy. To be successful in improving outcome, FLT3 inhibitors will need to be efficacious in targeting this small population of cells rather than simply reducing the bulk of the leukaemia. Encouragingly, there is some data that lestaurtinib can prevent the engraftment of FLT3/ITD mutated AML blast cells in a mouse model, suggesting that this inhibitor may indeed have the desired effect (Levis *et al*, 2005a).

In view of the limited clinical responses seen with lestaurtinib monotherapy (Knapper *et al*, 2006a, Smith *et al*, 2004), it is likely that any therapeutic role for lestaurtinib will be in combination with conventional chemotherapy. The data presented in this chapter demonstrates that the level of cell kill induced by lestaurtinib was synergistic with cytarabine, regardless of mutational status, and the degree of synergy observed was not statistically different between the 3 groups.

One concern with regards to the combination of FLT3 inhibitors with conventional chemotherapy agents is that inhibition of FLT3 is likely to slow down cell proliferation in AML blasts with activated FLT3, thereby reducing the efficacy of cell-cycle dependent chemotherapeutic agents such as cytarabine. Studies using cell line models have suggested that the sequence of administration of lestaurtinib in combination with conventional chemotherapy is important to achieve a synergistic effect of the two drugs (Levis *et al*, 2004). In these experiments, lestaurtinib pre-treatment of Ba/F3 cells transduced with a FLT3/ITD led to relative resistance to the effects of cytarabine. However, these results should be interpreted with caution for a number of reasons. Firstly, a cell line model system is biologically very different from primary AML cells and the impact of sequential administration of lestaurtinib and cytarabine on primary AML cells was not addressed. Studying the impact of the sequential incubation of lestaurtinib and cytarabine on primary AML cells *in vitro* is complicated by the decrease in viability with time of primary AML cells in liquid culture. Interpretation of such *in vitro* studies is therefore inevitably limited as it relies on a comparison between a control population of cells with inexorably declining viability and the impact of *in vitro* agents on the rate of this decline. Whilst this is clearly far removed from the *in vivo* treatment of AML in patients, it may nevertheless produce data that are more relevant than murine cell line studies. A further limitation of the above study relates to the longer period of exposure of the lestaurtinib pretreated Ba/F3 cells to lestaurtinib (72 hours versus 48 hours) which would lead to a reduced viability of the control cells. As these data were expressed as percentage cell viability relative to the control cells, artificial skewing of the curves and potential misinterpretation of the combination index could result.

In the work presented in this chapter, the impact of sequential administration of cytarabine and lestaurtinib was studied in 6 primary AML cases. The cytotoxic impact of cytarabine followed by lestaurtinib treatment was slightly greater than the two drugs in the opposite sequence (%OD, 46% versus 56%). This would support a possible antagonistic interaction of the two drugs when lestaurtinib is used first. However, the cytotoxic impact of cytarabine as a single agent was less when given in the second 48 hours compared to the first (%OD, 74% versus 57%), suggesting that the above effect does not relate to lestaurtinib. It may be that the blast cells which survive longer in liquid culture are less

sensitive to cytarabine-induced toxicity. Furthermore, pre-treatment of blast cells with lestaurtinib for the first 48 hours followed by a combination of cytarabine and lestaurtinib induced increased cytotoxicity compared to blast cells cultured in R10 alone for the first 48 hours followed by a combination of the two agents (%OD, 48% versus 65%). There is no evidence from these experiments, therefore, that pre-treatment with lestaurtinib reduces the cytotoxic effect of cytarabine on AML blast cells *in vitro*. This is an important question to adequately answer as it may be that concurrent administration of FLT3 inhibitors and chemotherapy may be beneficial, as inhibition of anti-apoptotic pathways may be more important *in vivo* than any possible detrimental impact because of an anti-proliferative effect. This may be particularly relevant for the leukaemic stem cell population, which are likely to be more quiescent than the bulk population of leukaemic cells.

Clinical studies are currently underway combining FLT3 inhibitors with conventional chemotherapy. One trial randomised patients with relapsed, FLT3 mutated AML to lestaurtinib with conventional chemotherapy or chemotherapy alone (Levis *et al*, 2005b). In this trial lestaurtinib and cytarabine were not administered concurrently. Of 17 patients randomised to receive lestaurtinib and chemotherapy, 10 achieved a response (5 CR) compared to 4 of 17 randomised to receive chemotherapy alone (2 CR). Another study combined PKC412 with conventional chemotherapy in patients with untreated AML, regardless of FLT3 mutation status (Stone *et al*, 2005a). Despite some problems with tolerability at a high dose level, with a dose reduction, the regime was well tolerated. An update of this trial (Stone *et al*, 2006) reported a CR rate of 69% in 26 FLT3/WT patients compared to 92% in 12 FLT3 mutated patients, with 7 of these 12 patients remaining in CR after a follow up of 3-15 months. These results are sufficiently encouraging, at least in FLT3 mutated patients, for these agents to be incorporated into larger, randomised trials of AML. For example, the UK MRC AML 15 trial has an amendment randomising FLT3 mutant-positive patients to receive lestaurtinib or not, in combination with conventional chemotherapy.

In conclusion, the data presented in this chapter suggests that in clinical trials of FLT3 inhibitors in adult AML, FLT3/TKD⁺ cases should not be differentiated from FLT3/WT cases. It should be emphasised that this does not imply that lestaurtinib should be restricted to FLT3/ITD⁺ cases, as an alternative approach would be for all patients to receive a FLT3

inhibitor, with the presence of a FLT3/ITD being used merely as a risk stratification variable. Nevertheless, it is encouraging that the cytotoxic effects of FLT3 inhibition appear to be most marked in the patient group where conventional chemotherapeutic approaches are most likely to fail.

CHAPTER 7: EXOGENOUS EXPRESSION OF FLT3 MUTANTS

IN CELL LINE MODELS AND HUMAN PRIMARY

HAEMATOPOIETIC STEM CELLS

7.1 Introduction

Following the observation that FLT3 is frequently mutated in AML, there has been considerable interest in developing experimental systems in order to understand the functional consequences of these mutations at a cellular level. These studies may provide insight(s) into mechanisms of leukaemogenesis and explain the adverse prognosis associated with FLT3/ITDs. If the latter is directly related to signalling events that arise as a result of mutation of this receptor, then characterising the downstream events may aid in understanding mechanisms of chemoresistance in AML and, more importantly, how to overcome them. Furthermore, if the different prognostic impact of FLT3/ITDs and FLT3/TKD mutations relates to differential signalling events, then direct comparison of the cellular consequences of these two mutations may also be relevant.

7.1.1 The role of FLT3 in normal haematopoiesis

The role of FLT3 in the growth and differentiation of normal haematopoietic cells has been widely studied. When used alone, FL was only a weak stimulator of CD34+ BM cells *in vitro*. When it was used in combination with other growth factors, however, FL synergistically enhanced the growth of primitive HSCs and more committed HPCs (Rusten *et al*, 1996), leading to a marked increase in the number of granulocyte-monocytic colonies formed in methylcellulose assays. A specific role in the differentiation of HSCs to B-lymphocyte progenitors has been suggested by *in vitro* studies (Veiby *et al*, 1996) as well as by *in vivo* mouse knockout studies of FLT3 (Mackarechtschian *et al*, 1995) or FL (Buza-Vidas *et al*, 2007). FL also appears to stimulate the proliferation and differentiation of early T-cells (Moore and Zlotnik 1997), and is a potent stimulator of dendritic cell development both *in vitro* and *in vivo*, leading to interest in its use to improve anti-tumour dendritic cell vaccination strategies (Dong *et al*, 2002). However, FL does not stimulate erythropoiesis or megakaryopoiesis (Ratajczak *et al*, 1995, Ratajczak *et al*, 1996), an observation that is

consistent with the lack of expression of FLT3 on megakaryocytic and erythroid progenitors (Adolfsson *et al*, 2005).

7.1.2 Signalling events downstream of FLT3/WT activation

Several groups had examined the intracellular signalling consequences of activating FLT3/WT before the identification of FLT3/ITDs in AML. As FL had not yet been discovered when these initial experiments took place, the first studies used chimeric molecules containing the extracellular domain of the CSF-1 receptor (c-FMS) fused to the transmembrane and cytoplasmic domains of murine Flt3. CSF-1 stimulation of Ba/F3 cells transduced with the chimeric receptor induced receptor dimerisation and autophosphorylation, led to IL-3 independent growth and tyrosine-phosphorylation of RAS GTPase-activating protein (GAP), phospholipase C- γ 1 (PLC γ 1), Vav, Fyn, Shc (SH2-containing sequence protein), src family tyrosine kinases and the p85 subunit of phosphatidylinositol-3 kinase (PI3K) (Casteran *et al*, 1994, Dosil *et al*, 1993). In co-precipitation assays it was shown that FLT3 directly associated with Grb2, PLC γ 1, the p85 subunit of PI3K, Shc and Src family tyrosine kinases. Furthermore, it was also observed that phosphorylation of p85 and Shc by FLT3 was cell-type-specific and differed between NIH 3T3 cells and Ba/F3 cells (Dosil *et al*, 1993).

The cloning of human FL in 1993 (see chapter 3) allowed the more direct study of FLT3 signalling pathways (Lavagna-Sevenier *et al*, 1998a, Lavagna-Sevenier *et al*, 1998b, Marchetto *et al*, 1999, Zhang and Broxmeyer 1999, Zhang and Broxmeyer 2000, Zhang *et al*, 1999). These studies mostly recapitulated the earlier observations using the chimeric receptor, with some notable differences. For example, human FLT3 does not bind directly to the p85 subunit of PI3K but rather activates PI3K by forming a complex with intermediaries such as GRB2, GAB2, SHIP, SHP2, CBL and CBLB. Through the phosphorylation of these intermediary proteins a number of key signalling pathways are activated including the PI3K pathway (Zhang and Broxmeyer 1999), the mitogen activated protein kinase (MAPK) pathway (Zhang *et al*, 1999) and signal transducers and activators of transcription such as STAT5A (Zhang *et al*, 2000), the latter by a JAK (Janus kinase) independent mechanism. Activation of the FLT3 kinase also leads to phosphorylation of SHIP (SH2-domain containing inositol phosphatase), which results in negative regulation of RAS and PI3K signalling (Marchetto *et al*, 1999).

7.1.3 Transduction of FLT3/ITDs in cell line models

FLT3/ITD induced ligand-independent activation of FLT3 was reported when plasmid constructs containing FLT3/WT or 5 different FLT3/ITDs were expressed in Cos7 cells (Kiyoi *et al*, 1998). Using immunoblotting, with or without immunoprecipitation, it was shown that FLT3/WT protein was not tyrosine phosphorylated in the absence of FL but addition of FL led to dimerisation and phosphorylation. FLT3/ITDs, however, were dimerised and tyrosine phosphorylated in the absence of FL, although in some cases the addition of FL further increased the level of tyrosine phosphorylation. It was also noted that the duplicated sequence of 51 different FLT3/ITDs almost always involved a tyrosine rich region between residues 589 and 599 (tyrosines at positions 589, 591, 597 and 599). However, by substituting tyrosine residues 589 or 591 to phenylalanine, it was shown that these tyrosine residues were not essential for FLT3/ITD-induced FL-independent activation. A further study demonstrated that FLT3/ITDs were capable of dimerising with and activating the FLT3/WT protein, even when the FLT3/ITD lacked a TKD domain and therefore had no intrinsic kinase activity (Kiyoi *et al*, 2002).

The downstream effects of FLT3/ITDs were investigated by introducing full-length WT and ITD forms of human FLT3 into the IL-3 dependent 32D and Ba/F3 murine cell lines by electroporation (Hayakawa *et al*, 2000). Upon deprivation of IL-3, the ITD-transduced cells continued proliferating whereas the WT-transduced cells showed weak proliferation in the presence of FL and rapidly died in the absence of cytokines. Both the ITD and FL-stimulated FLT3/WT led to phosphorylation of ERK1 (extracellular signal-regulated kinase) and ERK2, part of the MAPK signalling pathway. Intriguingly, however, whilst the ITD-transduced cells showed phosphorylation of STAT5A with associated DNA binding, this was absent in FL-stimulated WT-transduced cells. These findings were confirmed by another study (Mizuki *et al*, 2000), which also found that FLT3/ITDs retained dependence on FL stimulation for the phosphorylation of MAPK and AKT (protein kinase B), a key serine-threonine kinase. However, a different study showed that STAT5A was strongly activated by FL stimulation of FLT3/WT (Zhang *et al*, 2000). The explanation for these differences is unclear.

Subsequent studies have focused on anti-apoptotic pathways activated by FLT3/ITDs. An initial report demonstrated strong activation of protein kinase A (PKA) and ribosomal S6

kinase 1 (RSK1) in FLT3/ITD transformed Ba/F3 cells (Yang *et al*, 2005), equivalent to FL stimulation of FLT3/WT transformed cells. PKA and RSK1 activation in turn led to phosphorylation of the Bcl-2 family member BAD at serine112, thereby leading to an inhibition of apoptosis. Phosphorylation of BAD may also occur through an alternative pathway through upregulation of Pim-1 by FLT3/ITDs (Kim *et al*, 2005, Kim *et al*, 2006). However, another study demonstrated that in FLT3/WT-transduced 32D cells, anti-apoptotic pathways induced by FL stimulation were dependent on BAD phosphorylation for cell survival whereas FLT3/ITD-transduced cells maintained anti-apoptotic signalling by upregulating Bcl-X_L (an anti-apoptotic Bcl2 family member), even when BAD was dephosphorylated (Minami *et al*, 2003). A subsequent report showed that transduction of 32D cells with a FLT3/ITD led to very strong cytokine-independent phosphorylation and activation of AKT and consequent phosphorylation and inactivation of the proapoptotic protein Foxo3a (Brandts *et al*, 2005, Scheijen *et al*, 2004). Conversely, FL stimulation of FLT3/WT-transduced 32D cells only led to weak activation of AKT. Proliferation and viability of ITD-transformed 32D cells could be markedly reduced by cotransfection with double negative forms of AKT. Finally, other studies have shown an apparent cooperation of Wingless-type (Wnt) signalling pathways with FLT3/ITD signalling, with FLT3/ITDs inducing an increased expression of Frizzled-4, a receptor for Wnt ligands, and high- β -catenin protein levels in 32D cells transfected with a FLT3/ITD compared to ligand stimulated FLT3/WT cells (Tickenbrock *et al*, 2005).

7.1.4 Correlation of signalling studies in cell line models with primary AML blast cells

Studies of signalling pathways in murine haematopoietic cell lines may not, however, reflect the impact of FLT3/ITDs in primary leukaemic cells. Indeed, correlation of cell line studies with the activation of signalling pathways in primary AML blast cells has produced mixed results. One study failed to show any correlation between constitutive activation of FLT3 in primary AML blasts and the presence of a FLT3/ITD (Fenski *et al*, 2000). This study included 32 cases of AML, 4 of which were FLT3/ITD⁺. FLT3 protein was detectable by immunoprecipitation in 27 cases, and in 18 of these cases the FLT3 was autophosphorylated following 5 minutes stimulation with 100ng/ml FL (Fenski *et al*, 2000). There were only 3 cases which displayed ligand-independent autophosphorylation of FLT3 and only 1 of these cases had a FLT3/ITD. Three other cases were FLT3/ITD-positive but

only demonstrated FLT3 autophosphorylation in the presence of FL. In another study, however, all 12 cases studied (5 WT FLT3, 6 ITDs and 1 TKD) showed ligand-independent phosphorylation of FLT3 (Knapper *et al*, 2006b).

In terms of downstream signalling pathways, correlation with FLT3 status has also generally yielded mixed results. One study demonstrated STAT5 activation by Western blot analysis in 3 of 14 cases without a FLT3/ITD compared with 4 of 8 FLT3/ITD⁺ patients (Spiekermann *et al*, 2003). Furthermore, a study using a T-cell factor/lymphoid enhancer factor (TCF/LEF) reporter assay showed a borderline association between the presence of a FLT3/ITD and increased Wnt signalling in primary blast cells from 25 cases of AML (Simon *et al*, 2005). However, other studies observed either no correlation (Grandage *et al*, 2005), or an apparent negative correlation (Tamburini *et al*, 2007), between the presence of FLT3 mutations and PI3K activation.

These studies highlight the difficulty of studying signalling events in primary cells *ex vivo* that may have been cryopreserved and lack the growth factor stimulation they would normally receive *in vivo*. It is very difficult to know how closely these studies reflect the *in vivo* signalling consequences that arise as a result of FLT3 mutation when cells are within the bone marrow microenvironment.

7.1.5 Transduction of FLT3/TKDs in cell line models

The potential that mutations in the TKD of FLT3 could be constitutively activating was first demonstrated before the mutations were described in AML. Activating mutations in c-Kit were known to be located within the second TKD (Furitsu *et al*, 1993) and the authors therefore hypothesised that equivalent point mutations may also activate the FLT3 kinase. They transfected 32D cells with murine D838V FLT3, the equivalent to human D835V, and showed this led to autophosphorylation of FLT3 and factor independent growth (Fenski *et al*, 2000). Subsequently, FLT3/TKD mutations were detected in AML patients and a 32D cell model was used to demonstrate that a number of these mutations (D835Y, D835V, D835H, D835E, D835N and I836L+D) led to FLT3 autophosphorylation and proliferation in the absence of cytokines (Yamamoto *et al*, 2001).

Signalling events downstream of FLT3/TKD mutations (D835Y, Δ 836 and I836M+R) were reported in Ba/F3, 32D and HEK293 cells (Grundler *et al*, 2003) and compared with FLT3/WT and a FLT3/ITD. This study showed that TKD mutants induced factor-independent growth of Ba/F3 cells, autophosphorylation of FLT3 and significant phosphorylation of STAT5, equivalent to that of the FLT3/ITD. FL stimulated FLT3/WT cells did not show any STAT5 phosphorylation. This study therefore suggested that both ITDs and TKDs lead to similar signalling events in the absence of cytokines, although FLT3/ITD-transduced cells did proliferate more quickly than FLT3/TKD-transduced cells.

A subsequent report, however, suggested that there are important differences in the signalling events activated by the different FLT3 mutations (Choudhary *et al*, 2005b). They found that expression of a FLT3 ITD or TKD (D835Y) in 32D cells led to strong cytokine-independent autophosphorylation of FLT3 but that TKDs induced less pronounced autonomous growth than ITDs. Furthermore, FLT3/ITD-transduced 32D cells were able to form colonies in semi-solid media in the absence of cytokines, unlike FLT3/TKD-transduced cells or FL-stimulated FLT3/WT cells. FLT3/ITD cells were also more resistant radiation-induced apoptosis than FLT3/TKD cells as assessed by measuring cell viability after exposure to 5 Gy γ irradiation in the absence of cytokines. FLT3/ITD, TKD or FL-stimulated WT cells all had similar degrees of phosphorylated Shc and AKT. The FLT3/TKD and FL-stimulated WT 32D cells had less strongly activated ERK1 and 2 than FLT3/ITDs. Most strikingly, the FLT3/ITD cells strongly phosphorylated STAT5, had increased Pim-2 and CIS expression (both downstream targets of activated STAT5) and demonstrated suppression of C/EBP α and PU.1 expression, changes that were not apparent in FLT3/TKD and FL-stimulated WT 32D cells.

7.1.6 The impact of mutant FLT3 on haematopoietic cell differentiation

In addition to the above survival and proliferative signals, studies have also examined the impact of a FLT3/ITD on myeloid differentiation. In a G-CSF-induced model of cell differentiation in 32Dcl3 cells, transduction with a FLT3/ITD led to a block in differentiation, unlike FL-stimulation of FLT3/WT transduced cells (Zheng *et al*, 2002). It is possible that this relates to a FLT3/ITD-induced reduction in the level of expression of C/EBP ϵ (Zheng *et al*, 2002) and C/EBP α (Mizuki *et al*, 2003, Zheng *et al*, 2004a), or

inhibition of C/EBP α by phosphorylation (Radomska *et al*, 2006). Alternatively, suppression of other myeloid transcription factors by FLT3/ITDs, such as PU.1, may contribute to this differentiation block (Mizuki *et al*, 2003, Zheng *et al*, 2004a). Another study has suggested that FLT3/ITD-induced suppression of RGS2, a regulator of G-protein signalling, is important for the myeloid differentiation block (Schwable *et al*, 2005).

No studies have addressed the impact of FLT3/TKDs on cellular differentiation, although it is interesting to note that FLT3/TKDs, unlike ITDs, do not suppress C/EBP α or PU.1 expression (Choudhary *et al*, 2005b), and also lead to an increased expression of the tumour suppressor TSC-22 (transforming growth factor- β stimulated clone-22) which in turn promotes, rather than blocks, myeloid differentiation (Lu *et al*, 2007).

7.1.7 Transduction of FLT3 mutations into normal murine haematopoietic cells

The *in vivo* impact of FLT3/ITDs on murine haematopoiesis has been studied by transplanting mouse BM cells oncoretrovirally transduced with a FLT3/ITD into lethally irradiated syngeneic recipient mice. This led to the development of a lethal myeloproliferative disease with a latency of approximately 40-50 days (Kelly *et al*, 2002b). An ITD alone was insufficient to generate an overt leukaemic phenotype. This finding has been confirmed in other studies using a similar oncoretroviral transduction approach (Grundler *et al*, 2005). Furthermore, transgenic mice with the FLT3/ITD expressed either under a *vav* promoter or 'knocked-in' to the murine FLT3 locus (Lee *et al*, 2007, Lee *et al*, 2005) also display a similar phenotype. However, mice transplanted with BM cells from PML-RARA transgenic mice that were oncoretrovirally transduced with a FLT3/ITD did develop a leukaemia-like syndrome with many similar features to APL, a marked reduction in the latency time of disease onset and complete penetrance (Kelly *et al*, 2002a). This would suggest that these two different genetic events cooperate to cause leukaemia. Similar results have been obtained with combinations of FLT3/ITDs and the core binding factor- β /smooth muscle myosin heavy chain (CBF β -SMMHC) fusion gene (Kim *et al*, 2007) as well as the AML1-ETO fusion gene (Schessl *et al*, 2005).

The impact of FLT3/TKDs (D835Y and I836M+R) has also been examined in a murine transplantation model and compared with a FLT3/ITD (Grundler *et al*, 2005). In freshly

harvested BM cells, all mutant-transduced cells had activated AKT, ERK1 and STAT5. In BM cells starved for 4 hours, however, FLT3/ITD-transduced cells demonstrated stronger phosphorylation of STAT5 and ERK than FLT3/TKD-transduced cells. Phosphorylation of AKT was similar between the ITD and TKDs. Mice transplanted with FLT3/TKD-transduced BM, however, developed B and T-cell lymphoproliferative disorders with a longer latency, in contrast to the ITD-induced MPD with a latency of approximately 50 days.

7.1.8 Transduction of FLT3 mutations into normal human haematopoietic cells *in vitro*

Given the key role of FLT3 mutations in the pathogenesis of AML, and the potential limitations of murine models of human leukaemogenesis, studies of the impact of FLT3 mutations in human haematopoietic cells are of considerable importance. It is surprising therefore, that only a limited number of studies have been reported on human HSCs transduced with FLT3/ITDs. One study used oncoretrovirally transduced umbilical cord blood (UCB) CD34+ cells with a GFP-tagged vector or FLT3/ITD (Chung *et al*, 2005). The ITD-transduced cells demonstrated increased proliferation in stromal co-culture assays, as demonstrated by a 4-fold increase in the GFP-positive fraction of ITD-transduced cells, but no change in the GFP-positive fraction of vector-transduced cells over the course of 5 weeks. Interestingly, these cells showed a dramatic reduction in monocytic markers and an increase in erythroid lineage markers. Furthermore, using *in vitro* progenitor assays, an increase in erythroid colonies was also reported, although this was surprising in view of the lack of any role for FL in stimulating erythroid colonies (see above). However, given that FLT3 was expressed non-physiologically under the control of a constitutive promoter, caution is required in the interpretation of these results. In an additional experiment, STAT5A was shown to be activated in the FLT3/ITD- but not FLT3/WT-transduced cells. Similar results were observed in another study using an oncoretroviral transduction approach (Moore *et al*, 2007), which also showed that transduction of UCB CD34+ cells with a constitutively activated form of STAT5A led to a similar phenotype as FLT3/ITD transduction. A subsequent study used a lentiviral vector approach to transduce UCB CD34+ cells, which has the advantage of being cell-cycle independent and therefore not requiring cytokine pre-stimulation. This study again demonstrated an increased proliferation in FLT3/ITD-transduced cells compared to vector-transduced cells in liquid

and semi-solid cultures in the presence of SCF and thrombopoietin, a reduction in monocytic differentiation but a relative increase in erythroid markers during liquid culture (Li *et al*, 2007). Of note, all these studies used GFP-vector transduced cells rather than FLT3/WT-transduced cells as a control. No studies have transduced human CD34+ cells with FLT3/TKD mutations.

7.1.9 Aims

The work in this Chapter aimed to compare the functional impact of FLT3/ITDs and FLT3/TKDs at a cellular level in terms of proliferation and differentiation. To achieve this, lentiviral vectors were developed in order to express FLT3/WT and the different mutant forms of FLT3 in both murine and human cell lines and in primary HSCs.

7.2 Materials and methods

7.2.1 Cloning of FLT3 into an MSCV lentiviral vector

The parental plasmid constructs containing full length FLT3 with a D835Y mutation and a FLT3/ITD were kindly provided by Professor A. Khwaja (Department of Haematology, UCL). The ITD was a 57 bp duplication with a 3 bp insertion within the juxtamembrane domain of FLT3 and contained 4 tyrosine residues within the duplicated segment, residues 589, 591, 597 and 599 (Figure 7.1).

The plasmids required for lentiviral production were a kind gift from Dr Nathwani, Department of Haematology, UCL. The third generation, self-inactivating vector plasmid had a GFP gene downstream of an internal ribosomal entry site (IRES) expressed under the regulation of a murine stem cell virus promoter. This vector plasmid had *Bsr*GI restriction enzyme digest sites just 3' of GFP and 5' of the IRES as shown in Figure 7.2A. The FLT3/ITD and FLT3/TKD plasmids had *Bsr*GI restriction enzyme digestion sites upstream of FLT3 and 3' of GFP as shown in Figure 7.2B. To subclone the TKD and ITD sequences including the IRES-GFP into the lentiviral plasmid, approximately 1 µg of each plasmid DNA was digested with *Bsr*GI in a 20 µl reaction for 4 hours at 37°C according to the manufacturer's recommended conditions. Digested products were separated by 1% agarose gel electrophoresis and the required band was cut out of the gel and purified using

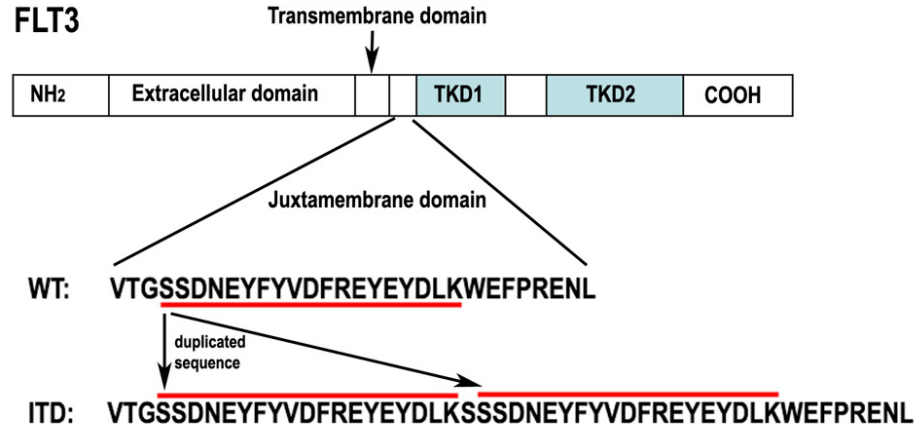


Figure 7.1: Duplicated segment of the juxtamembrane domain of the FLT3/ITD vector showing the inserted 20 amino acid sequence that resulted from a 57 bp tandem duplication and 3 bp insertion.

QIAquick Gel Extraction Kit (Qiagen, Crawley, West Sussex, UK). The purified and digested plasmid and FLT3-IRES-GFP insert were then ligated at 1:1, 1:3 and 1:5 molar ratios (plasmid:insert) using Promega T4 DNA Ligase (Promega, Southampton, UK) according to the manufacturer's recommended conditions. Ligated products were transformed into DH5 α competent bacteria by adding 5 μ l of the ligation reaction to 50 μ l of DH5 α competent bacteria and incubating on ice for 30 minutes. The cells were then heat-shocked at 37°C for 30 seconds and returned to ice for 2 minutes. Following this, 200 μ l of SOC medium was added and the cells incubated on a shaker for 60-90 minutes at 37°C. Cells were then spread onto LB-agar plates with carbenicillin added to 50 μ g/ml final concentration and incubated overnight at 37°C. Colonies were plucked the next day, transferred to 5 mls of LB medium containing carbenicillin and grown overnight. Bacterial cells were then pelleted and plasmid DNA extracted using QIAprep Miniprep kit (Qiagen, Crawley, West Sussex, UK). Insertion of FLT3 in the correct orientation was confirmed by *EcoRV* and *HincII* digestion. Forward orientation insertion of the FLT3/ITD resulted in 4

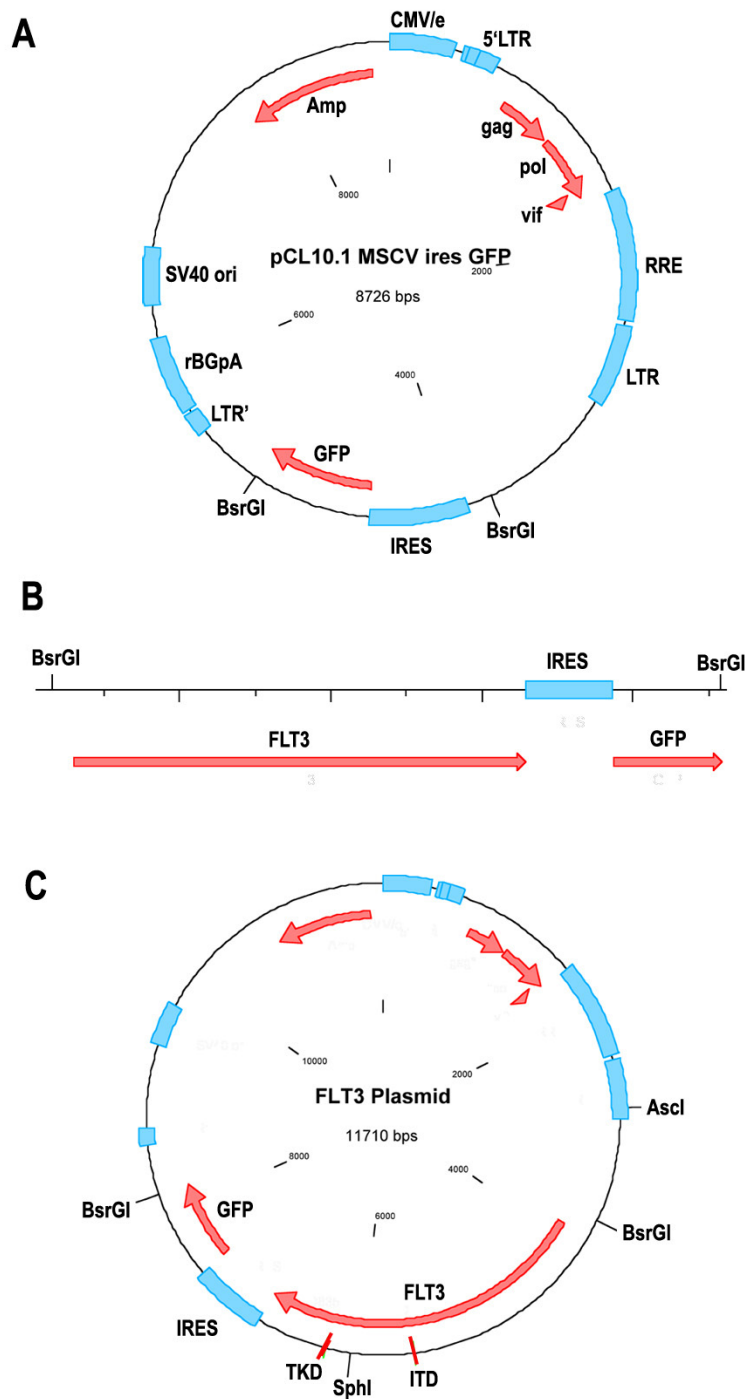


Figure 7.2: Plasmids used for FLT3 cloning. (A) The lentiviral-vector plasmid used with *BsrGI* sites marked. (B) The section of the plasmid containing FLT3 upstream of an IRES-GFP cassette with flanking *BsrGI* sites marked. (C) The resulting FLT3 plasmid with the sites of the ITD or TKD mutation marked. The *AscI* and *SphI* sites used to generate FLT3/WT are shown.

bands at 5304, 2672, 2220 and 1574 bp whereas for the D835Y, only 3 bands were present at 7916, 2220 and 1574 bp. FLT3/WT was not available in a plasmid and was therefore generated using *SphI* and *AscI* restriction enzyme digestion sites (shown in Figure 7.2C) in order to cut, gel extract and religate the WT segments of the FLT3/TKD and FLT3/ITD constructs. Thus 4 different plasmids were developed:

- **Vector:** GFP only
- **WT:** GFP-tagged FLT3/WT
- **TKD:** GFP-tagged D835Y FLT3/TKD
- **ITD:** GFP-tagged FLT3/ITD

DNA of the constructs and plasmids required for electroporation and lentivirus production was generated using Qiagen Megaprep kits (Qiagen, Crawley, West Sussex, UK).

7.2.2 Haemopoietic cell lines

32D clone 3 (32Dcl3) cells, a growth factor dependent murine myeloid cell line (Greenberger *et al*, 1983), and Ba/F3 cells, an IL3 dependent murine bone marrow derived pro-B cell line (Palacios and Steinmetz 1985) were grown in RPMI containing 10% fetal calf serum (R10) and 1% antibiotic/antimycotic solution (Sigma-Aldrich) in the presence of 10 nM murine IL3 (mIL3) in 5% CO₂ in a humidified incubator at 37°C. Cells were maintained at a density of approximately 1×10^5 /ml and were split 3 times per week.

The NB4 (Lanotte *et al*, 1991) cell line, derived from a case of human APL, was grown in R10 and 1% antibiotic/antimycotic solution without the addition of IL3. This cell line is FLT3/WT (Quentmeier *et al*, 2003), which was confirmed by direct sequencing of gDNA.

7.2.3 Electroporation of plasmid constructs into 32Dcl3 cells

32Dcl3 cells were transduced with the above plasmids by electroporation. Cells were resuspended in R10 at a density of $1-2 \times 10^5$ /ml and cultured overnight. The next day, they were washed, resuspended at a density of $20-40 \times 10^6$ /ml in 0.5 ml of phenol red-free medium containing 10% FCS, 10% WEHI-conditioned medium and 20 µg of one of the plasmids, and electroporated at 250 V and 960 mF. They were left for 5 minutes to recover and then resuspended in 15-20 ml of R10 and returned to the incubator. The following day, live cells were harvested by Ficoll-Hypaque centrifugation and cultured in 10 nM mIL3.

After 1 week, GFP-positive cells were selected by fluorescence activated cell sorting (FACS) on an EPICS, Beckman-Coulter flow cytometer under sterile conditions.

7.2.4 Lentiviral production

Third generation self-inactivating lentivirus was produced using a previously described 4-plasmid transient transfection method (Hanawa *et al*, 2002). 293T cells were seeded onto 10cm tissue culture plates at a density of approximately 0.3×10^6 /ml in 10 mls of DMEM containing 10% fetal calf serum (D10) and 1% antibiotic/antimycotic solution and placed in 5% CO₂ in a humidified incubator at 37°C. After 24 hours, the cells were approximately 90% confluent. For each plate, a mixture of 10 µg of the required plasmid, 7.5 µg of packaging plasmid (KGP1.1R), 2 µg of helper plasmid (RTR2), 2 µg of envelope plasmid (VSVG) and 50 µl of 2.5M CaCl₂ was made up to 500 µl with water. This was slowly mixed with an equal volume of 2 x HEPES-buffered saline whilst vortexing and then immediately mixed with 9mls of D10. The medium was removed from each 10cm plate and replaced with 10mls of the plasmid/D10 medium. The cells were then returned to the incubator.

After 24 hours, transduction was assessed using microscopy under UV light to detect GFP-expressing cells. The cells were then washed twice with 10 mls of PBS and fresh D10 medium added. After a further 24 hours of culture, the medium was harvested from each plate and filtered through a 0.2 µm filter. The lentiviral rich-medium was used fresh where possible, otherwise it was stored at -40°C.

In order to concentrate virus preparations for CD34+ cell transductions, viral supernatants were spun at 50000 g (27000 rpm) for 90 minutes at 4°C. The supernatant was then carefully decanted off under sterile conditions and 50 µl of X-vivo10 medium (Cambrex) added per tube. After 30 minutes the virus was fully resuspended by pipetting, transferred to a sterile eppendorf tube, then spun for 10 minutes at 1000 g (4000 rpm) to remove any cell debris. The supernatant was used fresh or aliquoted to cryovials and stored at -80°C.

7.2.5 Biological titre of lentiviral supernatants

Using a six well plate, HeLa cells were plated at a concentration of 1×10^4 cells per well in 2 mls of D10. After 24 hours, the medium was replaced with 1 ml fresh D10 containing 5 μ l, 50 μ l or 500 μ l of viral supernatant and 2 μ l of 1000x polybrene (final concentration 4 μ g/ml) and culture continued for 48 hours. The % GFP of HeLa cells was then measured by flow cytometry and the estimated titre of the virus calculated as follows:

$$\text{Viral titre} = \% \text{ GFP} \times 100,000 \times \text{dilution factor}$$

$$\text{Dilution factor} = 1000/\text{volume of viral supernatant added } (\mu\text{l})$$

7.2.6 Lentiviral transduction of haemopoietic cell lines

To transduce cell lines, cells were incubated in a 6 well plate at a density of 5×10^4 /ml in either R10 (Ba/F3) or D10 (NB4) in the presence of 4 μ g/ml polybrene and the specific viral supernatants at a multiplicity of infection (MOI) of 10 viral particles per cell for NB4 cells and 2 for Ba/F3 cells. After 24 hours the cells were washed and the medium replaced with R10, with the addition of 10 nM mIL3 for Ba/F3 cells. After a further 48 hours the cells were assessed for GFP positivity and GFP-positive cells selected by FACS.

7.2.7 Assessment of cell proliferation

To assess cell survival and proliferation, 32Dcl3 cells were washed and resuspended in R10 at a density of 1×10^5 /ml in the presence of cytokines and/or lestaurtinib as indicated. Cell number and viability were measured every 24 hours using trypan blue exclusion. Cell number was re-adjusted following each cell count to 1×10^5 /ml in R10.

Proliferation rate of the other cell lines was assessed using propidium iodide staining (Chapter 2.1.10) and flow cytometry. Cells were washed and re-suspended at a density of 5×10^4 /ml in R10 containing appropriate concentrations of cytokines and/or lestaurtinib as indicated. Cell number and viability were assessed every 2-4 days (depending on the rate of proliferation of the cells) by staining the cells with propidium iodide (see Chapter 2) and calculating the number of live cells relative to a known concentration of calibration beads. An example of this is shown in Figure 7.3. Cell density was re-adjusted following each cell

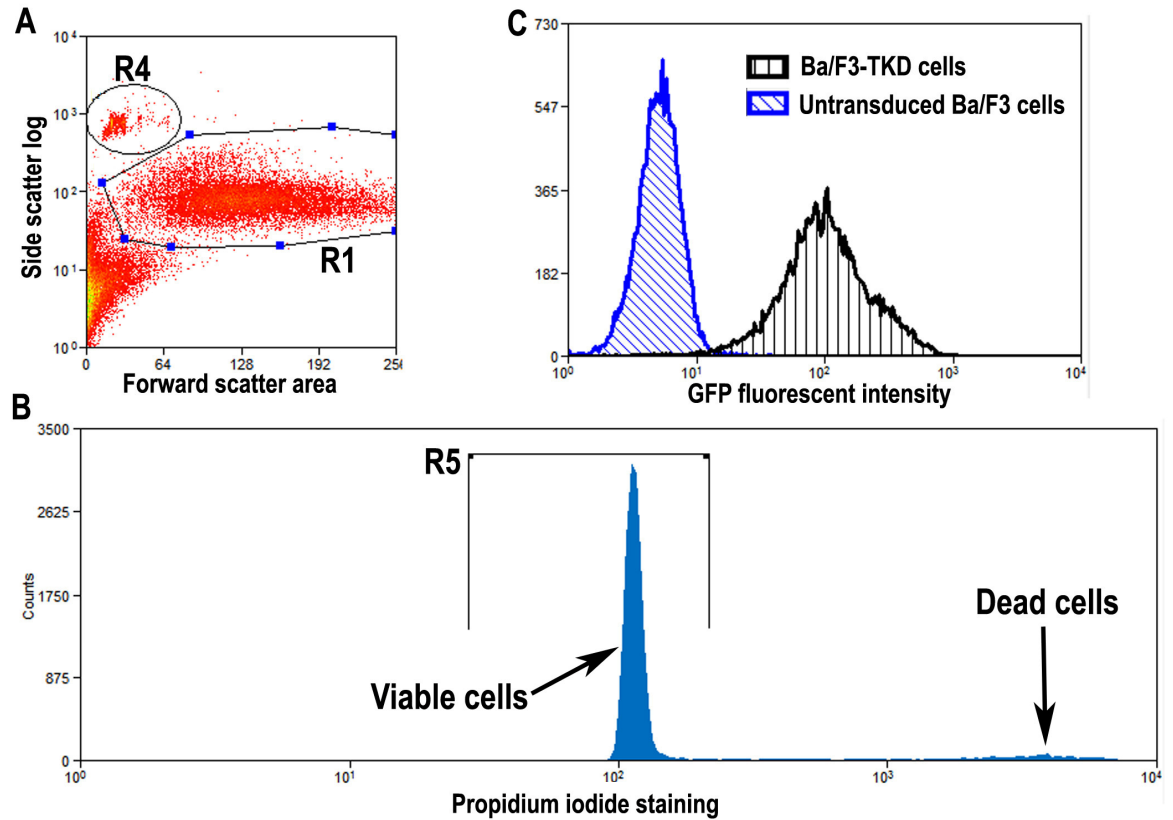


Figure 7.3: Assessment of cell number and viability using flow cytometry with propidium iodide staining and calibration beads. (A) The beads were added at a known density (x beads/ml) and quantified in gate R4 using a side scatter (SS) versus forward scatter (FS) plot. Ba/F3-TKD cells of unknown density (y cells/ml) were quantified by gating the cells on a SS/FS plot (gate R1) and **(B)** then assessing viability of the gated cells according to strength of PI staining (viable cells gated in R5). The density of viable cells was calculated as $y = x(R5/R4)$. **(C)** The Ba/F3-TKD cells were also gated to a histogram showing the relative GFP fluorescence (untransduced Ba/F3 control).

count to 5×10^4 /ml. The doubling time for each cell line was calculated using logistic regression and results are presented as R^2 and standard error of the mean (SEM).

7.2.8 Cytotoxicity assays

The effect of lestaurtinib was assessed by incubating cells at a density of 4×10^5 /ml in the presence of varying concentrations of the cytotoxic agent in a total volume of 100 μ l R10 or

D10 in 96 well plates. After 48 hours, 20 μ l of MTS tetrazolium compound (CellTitre 96[®] AQueous one solution cell proliferation assay reagent, Promega, UK) was added to each well and after a further 4 hours the absorbance at 490nm was measured and corrected for background absorbance in wells containing medium only. P-values are for the student T-test between two groups.

7.2.9 Detection of proteins by immunoblotting

Ba/F3 cells were washed twice in PBS and cultured for 24 hours in R10 containing 10 nM mIL3 for Ba/F3-vector cells, 20 ng/ml FL for Ba/F3-WT cells and R10 only for Ba/F3-TKD and ITD cells. Cells were then exposed to 0, 5 or 50 nM lestaurtinib for 6 hours. Cells were pelleted by centrifugation at 800 g (1500 rpm) for 5 minutes at 4°C, washed in ice cold PBS, mixed with 45 μ l lysis buffer per 1×10^6 cells and placed on ice for 30 minutes. The lysates were then clarified at 20000 g (13000 rpm) for 10 minutes at 4°C. The supernatant was aspirated, mixed with an appropriate volume of 5x loading buffer, boiled for 5 minutes and then used immediately or stored at -20°C until required. The lysates were run on SDS-PAGE gels and transferred to a nitrocellulose membrane as described in Chapter 2.

Membranes were incubated in the indicated primary antibody, at the manufacturers' recommended concentration, made up in 3% BSA, 0.1% PBS/Tween-20. All primary antibodies were incubated with the membrane overnight at 4°C. The primary antibody was removed, the membrane washed 3 times in 0.1% PBS/Tween-20 and then incubated with the appropriate horseradish peroxidase-linked secondary antibody diluted 1:10000 in 2% milk, 0.1% PBS/Tween-20 for 1 hour at room temperature. The membrane was then washed 3 times and protein bands detected by chemiluminescence (ECL or ECL-plus for phospho-specific antibodies) according to manufacturers' recommended guidelines. The membrane was then exposed to autoradiography film.

7.2.10 Assessment of intracellular phospho-proteins by flow cytometry

Ba/F3 cells were washed and incubated in the presence or absence of mIL3 and FL as for the immunoblotting. After 24 hours, lestaurtinib was added to a concentration of 10 nM as indicated and cells incubated for a further 6 hours. Approximately 1×10^6 cells were then

washed in ice cold PBS containing 1% BSA and resuspended in 250 μ l

Fixation/Permeabilisation solution (Bdbiosciences) for 20 minutes at 4°C. The cells were then washed twice in 1ml of 1x Perm/Wash™ buffer (Bdbiosciences.com), resuspended in 50 μ l of 1x Perm/Wash™ buffer containing 1 μ l of the primary antibody and incubated at 4°C for 30 minutes in the dark. The cells were then washed and resuspended in 50 μ l of 1x Perm/Wash™ buffer containing 5 μ l of the goat anti-mouse IgG antibody conjugated to allophycocyanin (APC) and incubated at 4°C for 30 minutes in the dark. The cells were then washed twice, resuspended in PBS containing 1% BSA and kept on ice until flow cytometric analysis. Control cells were treated in an identical fashion with the exception that the primary antibody was not added.

7.2.11 NB4 Cell Differentiation Experiments

Transduced NB4 cells were incubated at a density of 2×10^5 /ml in R10 in the presence or absence of 0.037 μ M, 0.111 μ M, 0.333 μ M or 1 μ M ATRA with or without 10 nM lestaurtinib for 4 days. The level of differentiation was assessed by flow cytometry using an APC-conjugated anti-CD11b antibody. Approximately 5×10^5 cells were washed twice in ice cold PBS and incubated with the antibody according to the manufacturer's recommended conditions (BD Pharmingen). Cells were washed twice with ice cold PBS prior to flow cytometric analysis. Results are expressed as the median fluorescent intensity (MFI) of APC.

7.2.12 Primary CD34+ Cells

Local ethical approval for the use of 'left-over' peripheral blood stem cell harvest samples was obtained prior to these studies. All studies were carried out following appropriate, generic informed consent for the use of these cells for research studies. CD34 cells were selected from peripheral blood stem cell harvests by a double round of magnetic cell sorting (MACS® cell separation columns and reagents, Miltenyi Biotec) according to manufacturers recommended protocol. Following this the percentage of CD34 +ve cells was >95% as measured by flow cytometry using a phycoerythrin conjugated anti-CD34 antibody (BD Pharmingen). Cells were either cryopreserved until required or were re-suspended at a density of 1×10^6 /ml in Stem Pro-34 SFM medium containing 20ng/ml SCF, 20 ng/ml FL, 45 ng/ml TPO and 10 ng/ml IL3 and placed in 5% CO₂ in a humidified

incubator at 37°C until lentiviral transduction later the same day. If frozen CD34+ cells were used they were thawed and cultured overnight in the above cytokines prior to viral transduction.

Approximately 2×10^5 cells were incubated in the minimum possible volume of concentrated lentiviral medium to provide a MOI of 50. Polybrene was not used as this led to a high level of cell death. The mix was incubated overnight in 5% CO₂ in a humidified incubator at 37°C then washed, resuspended in Stem Pro-34 containing the above cytokines and cultured for 72 hours. Following this, the cells were washed and cultured in Stem Pro-34 in the absence of cytokines for 24 hours. The cell density and percentage of GFP positive cells were then measured by flow cytometry (day 0). The number of viable cells was measured every 2-3 days for a total of 2 weeks by propidium iodide staining relative to a known concentration of calibration beads.

Following 7 and 14 days in the above cytokine free Stem Pro-34 liquid culture medium, the volume of medium estimated to contain 250 viable cells was added to 0.5 mls of methycellulose in the presence of 20ng/ml SCF, 20ng/ml FL, 20 ng/ml GM-CSF and 50ng/ml G-CSF and placed in 5% CO₂ in a humidified incubator at 37°C. The number of colonies was counted after 1 and 2 weeks.

7.3 Results

7.3.1 Confirmation of FLT3 expression using the cloned plasmid constructs

In order to confirm that the plasmid constructs containing the different forms of FLT3 led to expression of the FLT3 protein, they were transiently transfected into 293T cells using calcium phosphate. Transduction efficiency was over 90% for all 4 plasmids as assessed by GFP positivity by flow cytometry. Cell lysates were made and immunoblotting for FLT3 revealed very high levels of expression in the WT, TKD and ITD-transduced cells but absence of FLT3 protein in the vector-transduced cells (Figure 7.4).

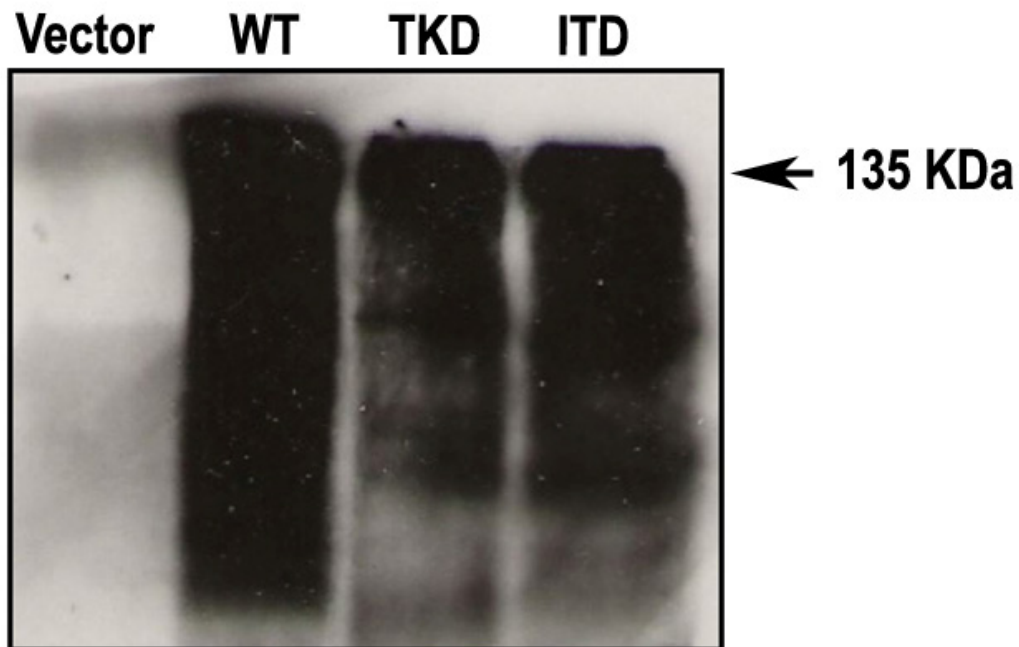


Figure 7.4: Detection of FLT3 protein (135 kDa) by immunoblotting in 293T cells transduced with the different FLT3 constructs.

7.3.2 Expression of FLT3 in haemopoietic cell lines

Following electroporation of 32Dcl3 cells, transduction efficiency was only 1-2% and two rounds of FACS sorting were therefore required in order to obtain a highly selected population of GFP-positive cells. Ba/F3 cells were transduced with lentiviral supernatants with an MOI of 2 as higher MOIs led to high levels of cell death. The transduction efficiency varied between 8% and 62%. Both 32Dcl3 and Ba/F3 cells were maintained in 10nM mIL3 until the start of each experiment. NB4 cells were transduced with lentiviral supernatants with an MOI of 10. High levels of transduction were achieved, between 78% and 99% as assessed by the percentage of GFP positive cells.

In each case, cells were selected according to approximately equal GFP fluorescent intensity as the level of GFP should directly correlate with expression of FLT3. This was confirmed by immunoblotting with anti-FLT3 antibody (Figure 7.5). As has been noted by

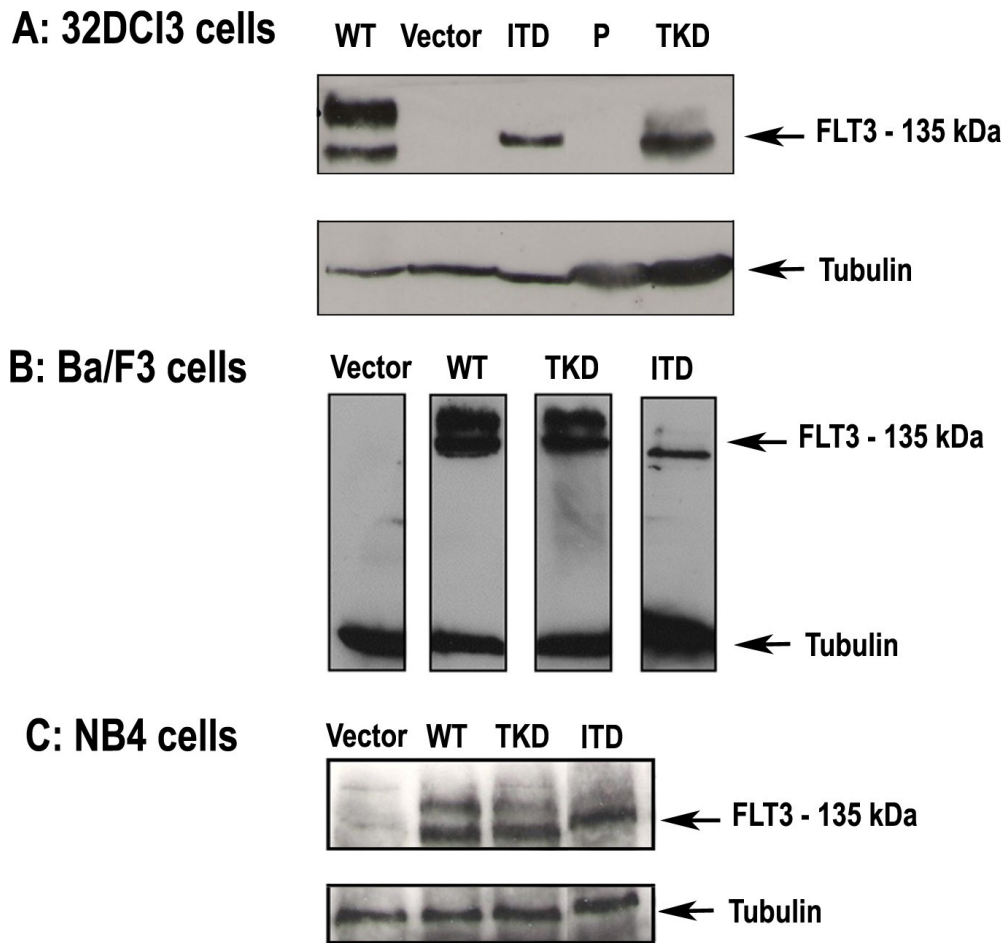


Figure 7.5: Western blotting studies to show expression of FLT3 protein in transduced cell lines. (A) 32Dcl3 cells; (B) Ba/F3 cells; and (C) NB4 cells. P indicates parental (untransduced) cells. Blots were probed with antibodies as specified.

others, the larger, glycosylated FLT3 band is weaker for FLT3/ITD and to a certain extent FLT3/TKD than for FLT3/WT (Choudhary *et al*, 2005b).

7.3.3 Impact of transduced FLT3 expression on the proliferation of haemopoietic cell lines

32Dcl3-vector cells proliferated rapidly in the presence of mIL3 whereas in the absence of cytokines the cells failed to proliferate and died within 3 to 4 days (Figures 7.6A, 7.7 and Table 7.1). The addition of FL did not increase survival of 32D-vector cells in the absence of mIL3 (Figure 7.7). Proliferation in the presence of mIL3 was not significantly impacted by the addition of 20 nM lestaurtinib.

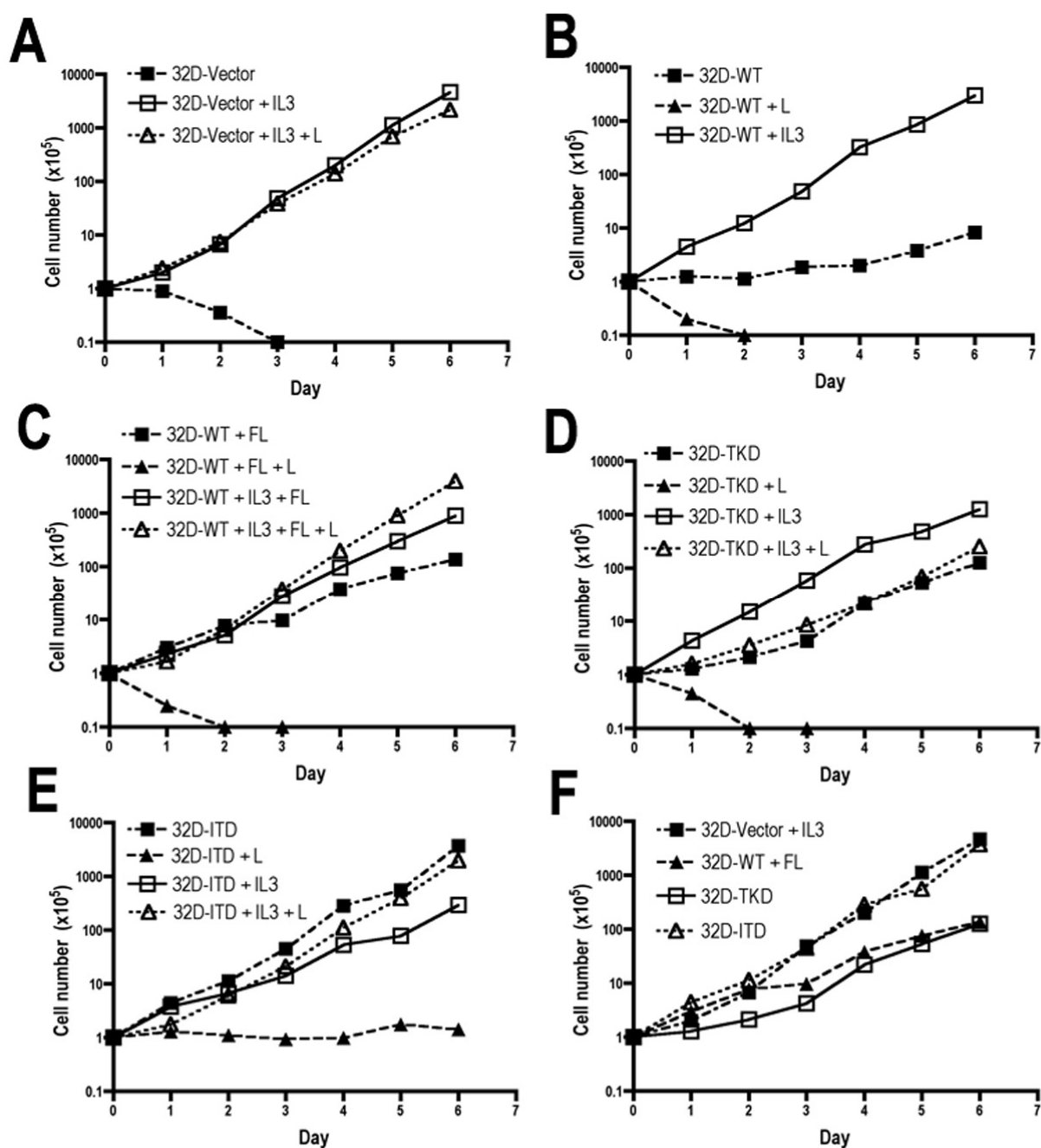


Figure 7.6: Proliferation rates of transduced 32Dcl3 cells. (A) 32Dcl3-vector, (B) 32Dcl3-WT, (C) 32Dcl3-WT + FL, (D) 32Dcl3-TKD, (E) 32Dcl3-ITD and (F) a comparison of 32Dcl3-vector + IL3, 32Dcl3-WT + FL, 32Dcl3-TKD + nil and 32Dcl3-ITD + nil. Cells were grown in the presence of 10 ng/ml murine IL3 (IL3), 20 nM lestauritinib (L) and 20 ng/ml FLT3 ligand (FL).

Table 7.1 Proliferation rates of transduced cell lines

	32Dcl3			Ba/F3			NB4		
	DT (hours)	SEM	R2	DT (hours)	SEM	R2	DT (hours)	SEM	R2
Vector	N/A	N/A	N/A	N/A	N/A	N/A	31.2	1.1	0.98
Vector + IL3	12.1	0.5	0.99	15.7	1.1	0.96	N/A	N/A	N/A
Vector + IL3 + L	12.5	0.5	0.99	17.5	1.2	0.95	N/A	N/A	N/A
WT	51.6	2.6	0.85	35.3	3.0	0.75	31.4	1.0	0.98
WT + IL3	12.3	0.4	0.99	13.8	0.6	0.99	N/A	N/A	N/A
WT + IL3 + L	N/A	N/A	N/A	14.5	0.8	0.98	N/A	N/A	N/A
WT + FL	20.5	1.0	0.99	22.6	1.7	0.91	N/A	N/A	N/A
WT + FL + IL3	14.1	0.56	0.99	14.4	0.8	0.98	N/A	N/A	N/A
WT + FL + IL3 + L	11.4	0.8	0.99	14.6	0.8	0.98	N/A	N/A	N/A
TKD	16.0	0.6	0.98	18.7	1.1	0.96	31.1	1.1	0.98
TKD + IL3	13.8	0.8	0.98	15.0	0.9	0.97	N/A	N/A	N/A
TKD + IL3 + L	17.9	1.3	0.98	15.9	1.2	0.95	N/A	N/A	N/A
ITD	12.4	0.6	0.99	14.4	0.9	0.97	30.7	1.0	0.98
ITD + IL3	18.4	0.7	0.98	14.8	0.8	0.97	N/A	N/A	N/A
ITD + IL3 + L	12.8	0.8	0.99	14.2	0.8	0.98	N/A	N/A	N/A

DT indicates doubling time; SEM, standard error of the mean for the doubling time; R2, the coefficient of determination for the logistic regression used to calculate the doubling time; L, lestaurtinib 20 nM; IL3, 10 ng/ml murine IL3; FL, 20 ng/ml FLT3 ligand; and N/A not applicable

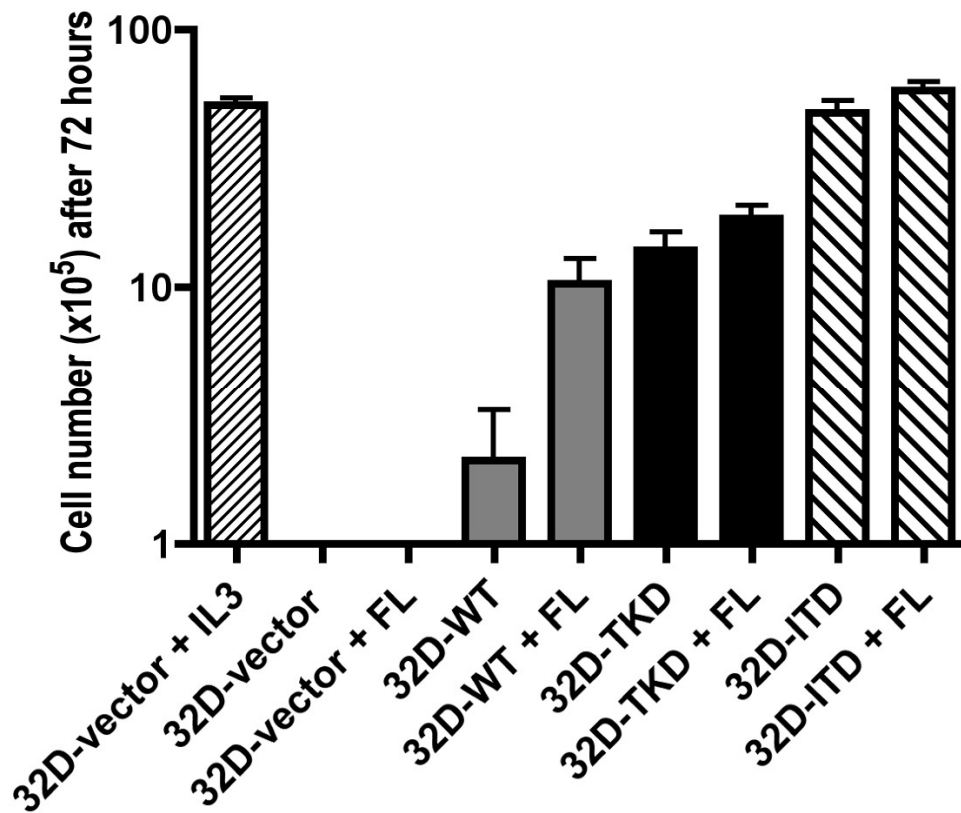


Figure 7.7: Impact of the addition of 20 ng/ml FL on the proliferation of 32Dcl3 transduced cells over 72 hours.

32Dcl3-WT cells proliferated at a similar rate to vector-transduced cells in the presence of mIL3 (Figures 7.6B, Table 7.1). In the absence of mIL3, FLT3/WT transduced cells retained viability but proliferated at a markedly reduced rate which was inhibited with the addition of 20nM lestaurtinib.

Addition of 20 ng/ml FL increased the proliferation rate of FLT3/WT transduced cells (Figure 7.6C, Table 7.1), however, this was still slower than that induced by mIL3. The FL-induced 32Dcl3-WT proliferation was inhibited by the addition of 20nM lestaurtinib. The presence of mIL3 in addition to FL significantly increased the proliferation of FLT3/WT transduced cells to the rate observed with mIL3 stimulated

vector-transduced cells and, surprisingly, the addition of 20nM lestaurtinib increased this rate of proliferation further.

In the absence of cytokines, 32Dcl3-TKD cells did proliferate, albeit at a slower rate compared with mIL3 stimulated 32Dcl3-vector cells (Figure 7.6D and Table 7.1), but nevertheless at an increased rate compared with 32Dcl3-WT cells grown in the presence of FL. The cytokine-free proliferation of 32Dcl3-TKD cells was inhibited in the presence of 20nM lestaurtinib. Addition of mIL3 to 32Dcl3-TKD cells led to a considerable increase in the rate of proliferation to a similar rate to that seen with 32Dcl3-vector cells but the proliferation was slower if 20nM lestaurtinib was present. The addition of 20 ng/ml FL to 32Dcl3-TKD cells led to a minor increase in the rate of proliferation (Figure 7.7).

32Dcl3-ITD cells also proliferated rapidly in the absence of cytokines (Figures 7.6E and Table 7.1) at a similar rate to mIL3-stimulated 32Dcl3-vector cells and significantly faster than cytokine free 32Dcl3-TKD cells. For example, the doubling time of 32Dcl3-ITD cells was 12.4 hours compared to 16 hours for 32Dcl3-TKD cells. The cytokine-free proliferation of 32Dcl3-ITD cells was also inhibited in the presence of 20nM lestaurtinib. Surprisingly, addition of mIL3 slowed the proliferation rate of 32Dcl3-ITD cells, an effect that was reversed with the addition of 20 nM lestaurtinib. The addition of 20 ng/ml FL to 32Dcl3-ITD cells led to a minor increase in the rate of proliferation (Figure 7.7).

As shown in Figure 7.8A-F, the results observed with transduced Ba/F3 cells were similar to those seen with 32Dcl3 cells. The addition of mIL3 to FL stimulated Ba/F3-WT and cytokine free Ba/F3-ITD cells, however, had no significant impact on the rate of cell growth.

As opposed to the marked effect of mutant FLT3 expression on the proliferation of mIL3-dependent murine cell lines, transduction of NB4 cells with WT or mutant FLT3 had no impact on the proliferation rate of this factor-independent, human cell line (Figure 7.9, Table 7.1).

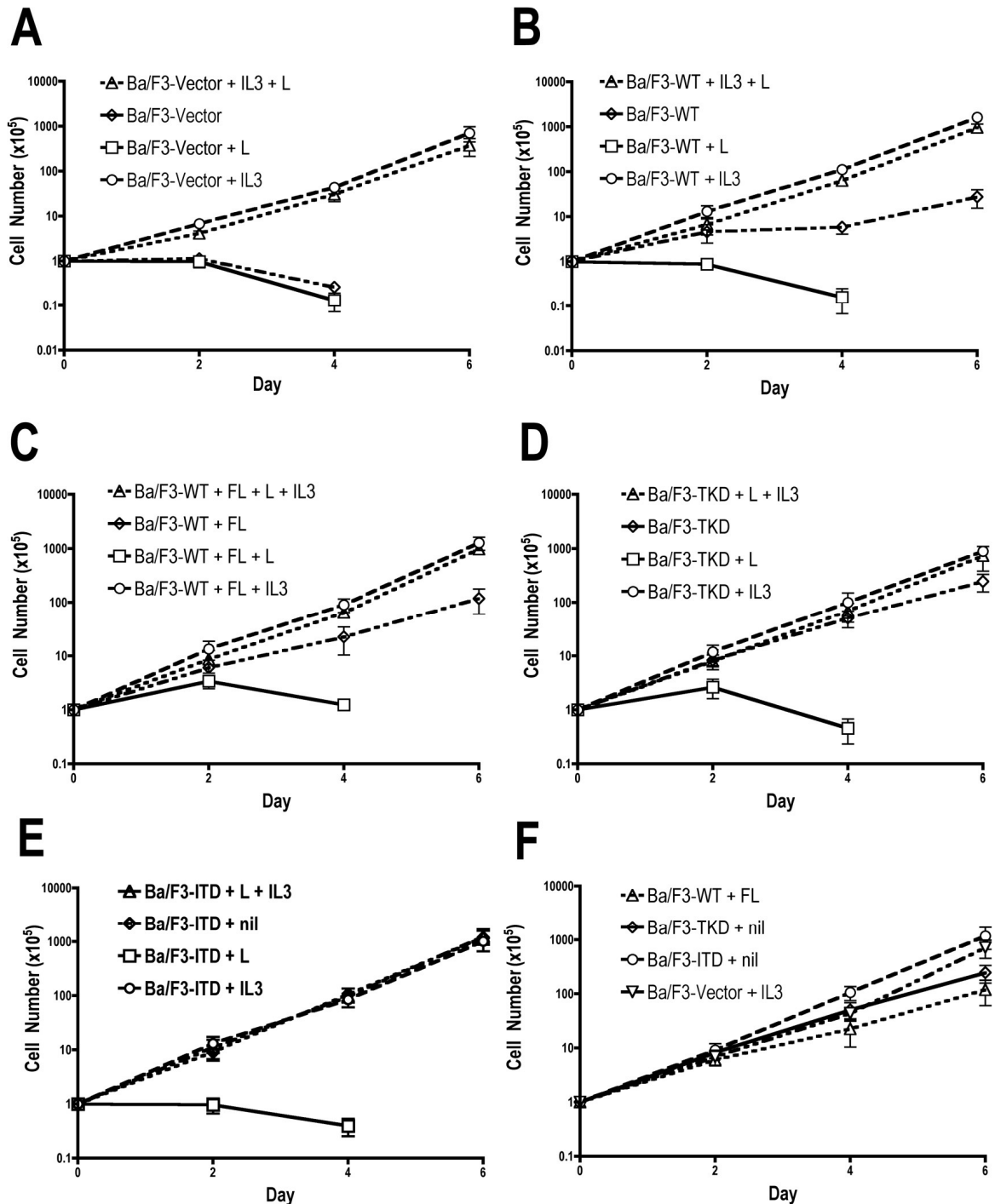


Figure 7.8: Proliferation rates of transduced Ba/F3 cells. (A) Ba/F3-vector, (B) Ba/F3-WT without FL, (C) Ba/F3-WT with 20 ng/ml FL, (D) Ba/F3-TKD, (E) Ba/F3-ITD and (F) a comparison of Ba/F3-vector + IL3, Ba/F3-WT + FL, Ba/F3-TKD + nil and Ba/F3-ITD + nil. IL3 indicates that cells were grown in the presence of 10 ng/ml murine IL3; L indicates that cells were grown in the presence of 20 nM lestaurtinib.

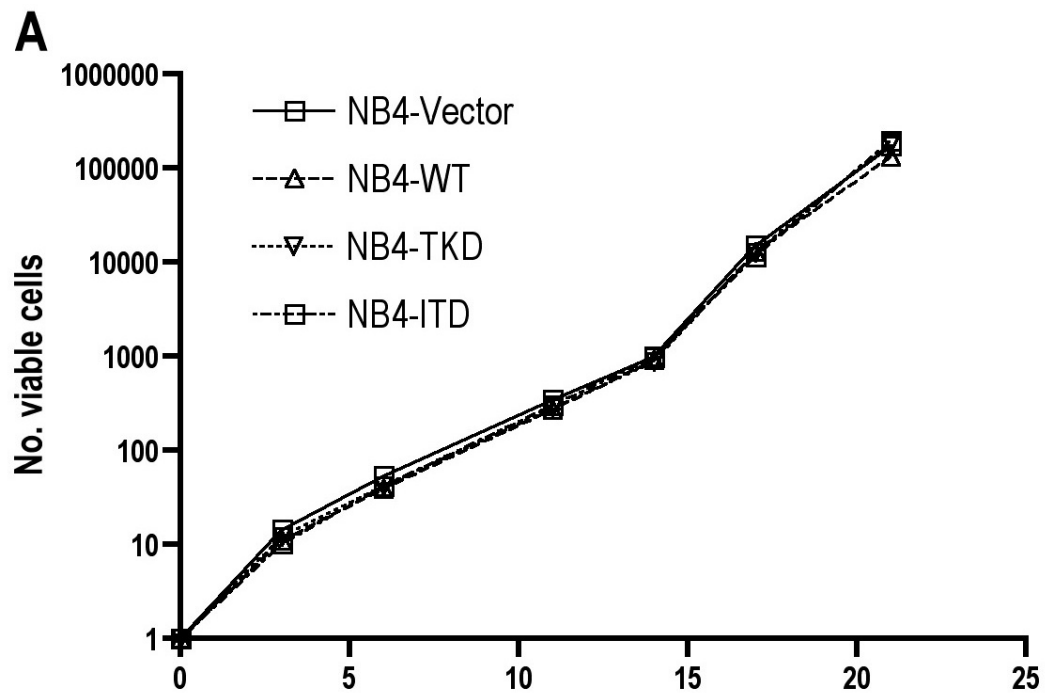


Figure 7.9: Proliferation rates of NB4 and cells transduced with the different constructs.

7.3.4 Effect of cytotoxic agents on FLT3 expressing cell lines

MTS assays of 32Dcl3 cells transduced with a TKD or an ITD and incubated for 48 hours with lestaurtinib showed a dose-dependent cytotoxic effect (Figure 7.10A).

However, at lower concentrations of lestaurtinib, 32Dcl3-TKD cells were more sensitive than 32Dcl3-ITD cells. For example, at 1nM lestaurtinib, the mean %OD was 62% for 32D-TKDs and 95% for 32D-ITD cells (Figure 7.10A, $P=0.03$). The addition of 5 nM FL had little impact on the cytotoxic effect of lestaurtinib on 32Dcl3-ITD cells (Figure 7.10B) or 32Dcl3-TKD cells (Figure 7.10C).

Increased sensitivity to lestaurtinib-induced cytotoxicity was also seen with Ba/F3-TKD cells in comparison with Ba/F3-ITD cells (Figure 7.10D). Lestaurtinib at lower concentrations had little impact on Ba/F3-vector cells grown in the presence of IL3, although at a concentration of 25 nM the %OD was reduced to 81% suggesting that higher concentrations of lestaurtinib may also impact on IL3 signalling.

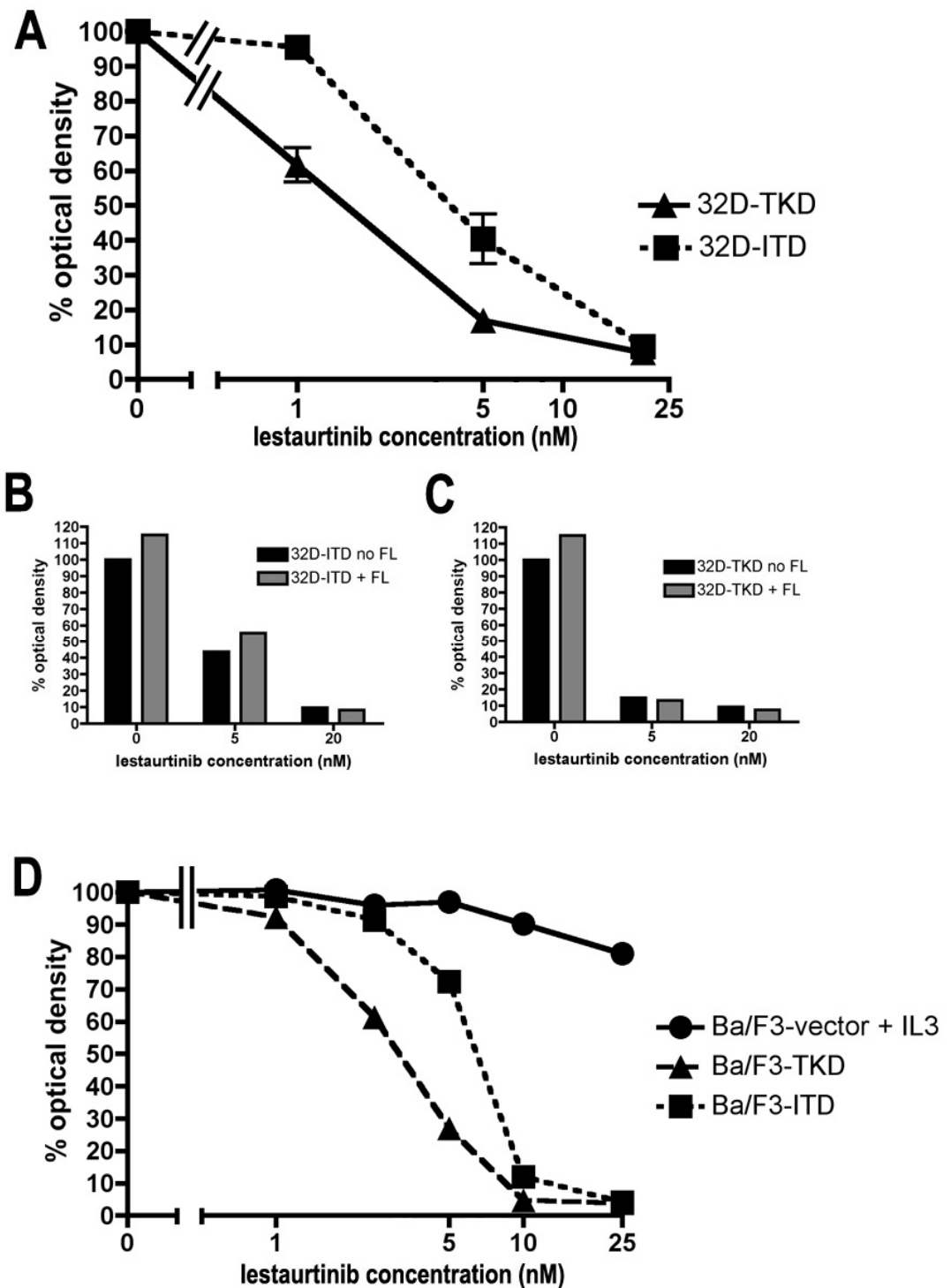


Figure 7.10: Cytotoxic effect of lestaurtinib on FLT3 transduced 32Dcl3 and Ba/F3 cells. (A) dose response of lestaurtinib in 32D-ITD and 32D-TKD cells; (B) impact of 20 ng/ml FL on the cytotoxic effect of lestaurtinib for 32D-ITDs and (C) 32D-TKDs; and (D) dose response of lestaurtinib in Ba/F3-vector cells grown in the presence of 10 ng/ml murine IL3 and cytokine-free Ba/F3-ITD and Ba/F3-TKD cells.

It is possible that the difference in %OD observed between TKD and ITD-transduced 32Dcl3 and Ba/F3 cells was due to a difference in the proliferation rate, rather than a cytotoxic effect. The percentage of dead cells following incubation in lestaurtinib for 24 hours was therefore assessed using propidium iodide staining. Ba/F3-vector cells grown in mIL-3 were not affected by the addition of lestaurtinib (Figure 7.11). With 10 nM lestaurtinib, the percentage of cell death was lower for Ba/F3-ITD cells than Ba/F3-TKD cells at 57% and 71% respectively (repeated in triplicate, $P=0.04$), consistent with a more marked cytotoxic effect on Ba/F3-TKD cells compared with Ba/F3-ITD cells.

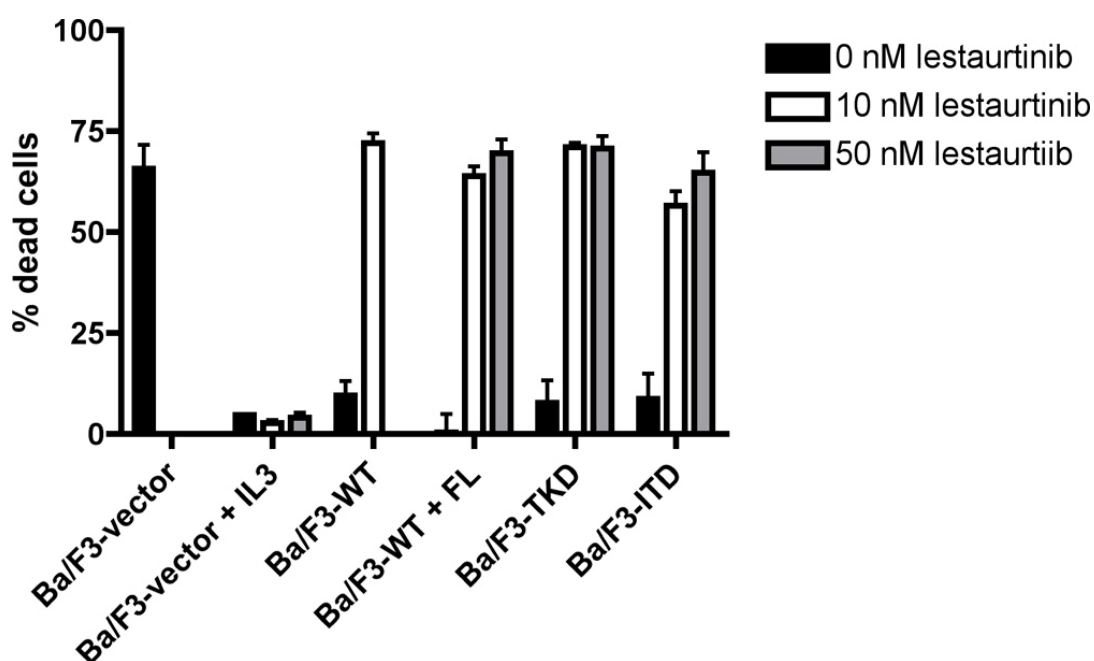


Figure 7.11: The impact of lestaurtinib on Ba/F3 transduced cells assessed by propidium iodide staining after 24 hours.

7.3.5 Signalling events in transduced Ba/F3 cells

The different proliferative rates arising from cells transduced with WT or mutant FLT3 suggest that signalling may differ between the different forms of FLT3. Therefore, the signalling pathways activated by the different FLT3 mutants or by mIL3 stimulation were investigated by immunoblotting and flow cytometry.

Ba/F3-vector cells grown in the presence of 10 ng/ml mIL3 showed marked activation of ERK, AKT and STAT5 that were not affected by the addition of up to 50nM lestaurtinib (Figure 7.12). Ba/F3-WT cells stimulated with FL showed activation of ERK and AKT but not STAT5. The ERK and AKT activation were inhibited by the

addition of 5nM lestaurtinib. Ba/F3-TKD cells showed weak STAT5 activation but strong activation of AKT, both of which were inhibited by lestaurtinib. In Ba/F3-ITD cells STAT5 was strongly activated, along with ERK and AKT. This activation was

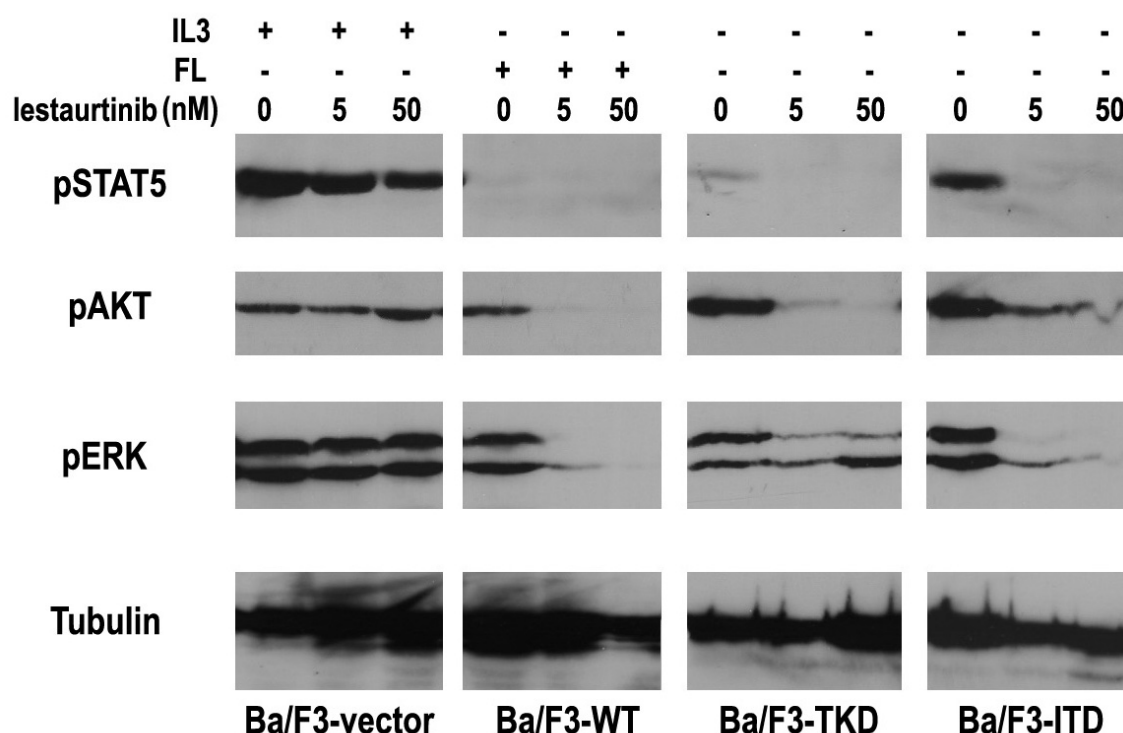


Figure 7.12: Immunoblotting of phospho-signalling proteins in transduced Ba/F3 cells. Cells were incubated for 24 hours in the presence or absence of 10 nM mIL3 or 20 ng/ml FL as indicated. Cells were then exposed to 0, 5 or 50 nM lestaurtinib for 6 hours following which cell lysates were made. Blots were probed with antibodies as specified.

inhibited by 5 nM of lestaurtinib, although, at this concentration, some residual AKT activation was seen which was reduced by 50 nM of lestaurtinib.

Intracellular signalling was also assessed by immunostaining and flow cytometry of fixed and permeabilised cells which allowed quantification of the relative level of activation of these signalling proteins. Median fluorescence intensity (MFI) was expressed relative to control cells where the primary antibody was not added. Three independent experiments were performed, a representative example for phospho-STAT5 is shown in Figure 7.13 and the mean MFI for all 3 experiments is shown in Figure 7.14A. Ba/F3-vector cells grown in the presence of mIL3 showed strong pSTAT5 fluorescence relative to control which was slightly reduced by pre-incubation of cells for 6 hours with 10 nM lestaurtinib and markedly reduced when cells were grown

in the absence of mIL3 for 24 hours. Ba/F3-WT cells incubated with 20ng/ml FL and Ba/F3-TKD cells showed only weak pSTAT5 fluorescence relative to control. This was further reduced by the addition of lestaurtinib. Ba/F3-ITD cells, however, showed

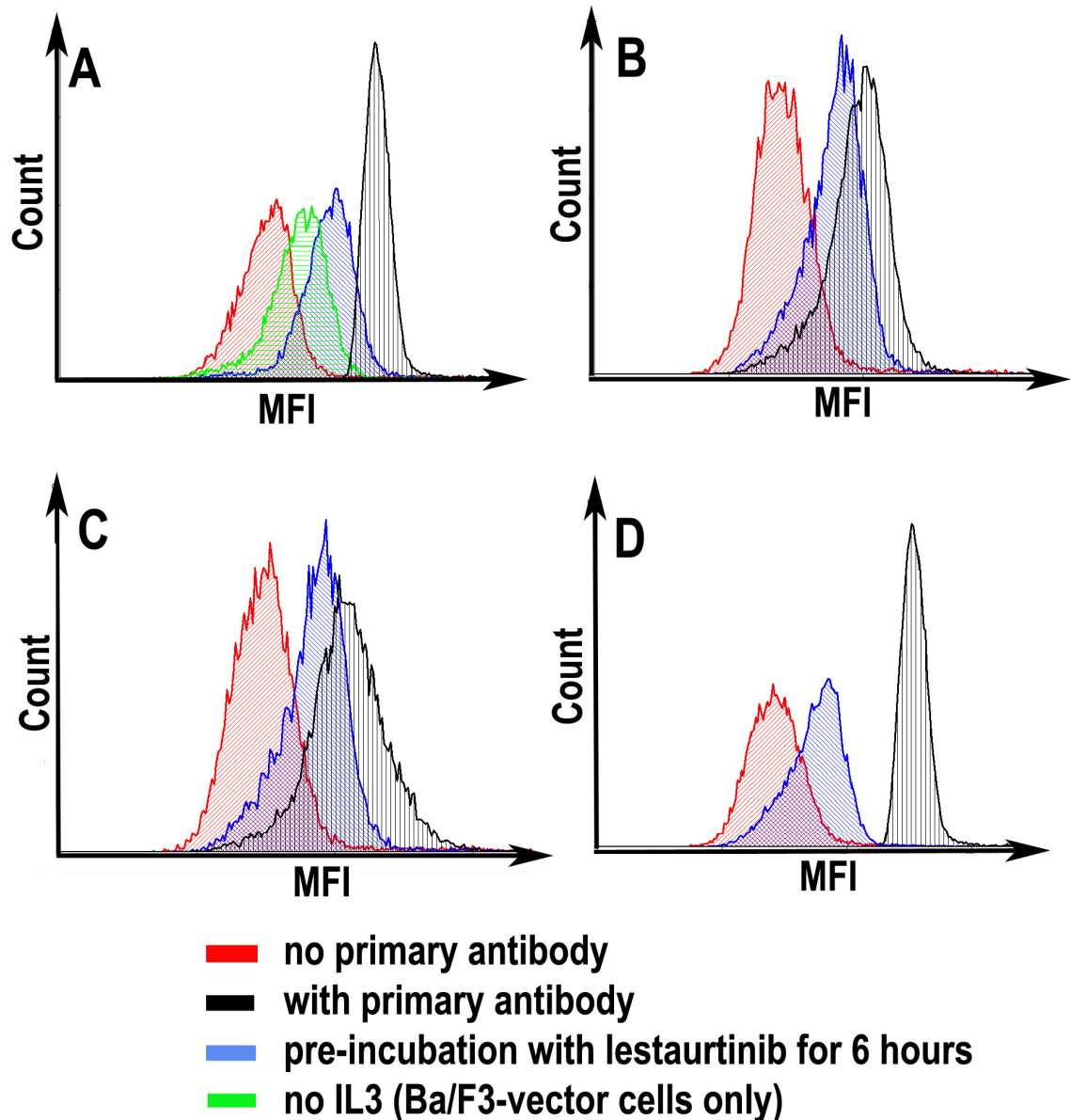


Figure 7.13: Assessment of intracellular phospho-STAT5 by flow cytometry of fixed and permeabilised transduced Ba/F3 cells. (A) Ba/F3-vector cells grown in the presence of 10 nM mIL3 unless labelled as otherwise (green histogram only); (B) Ba/F3-WT cells grown in the presence of 20 ng/ml FL; (C) cytokine-free Ba/F3-TKD cells; and (D) cytokine-free Ba/F3-ITD cells.

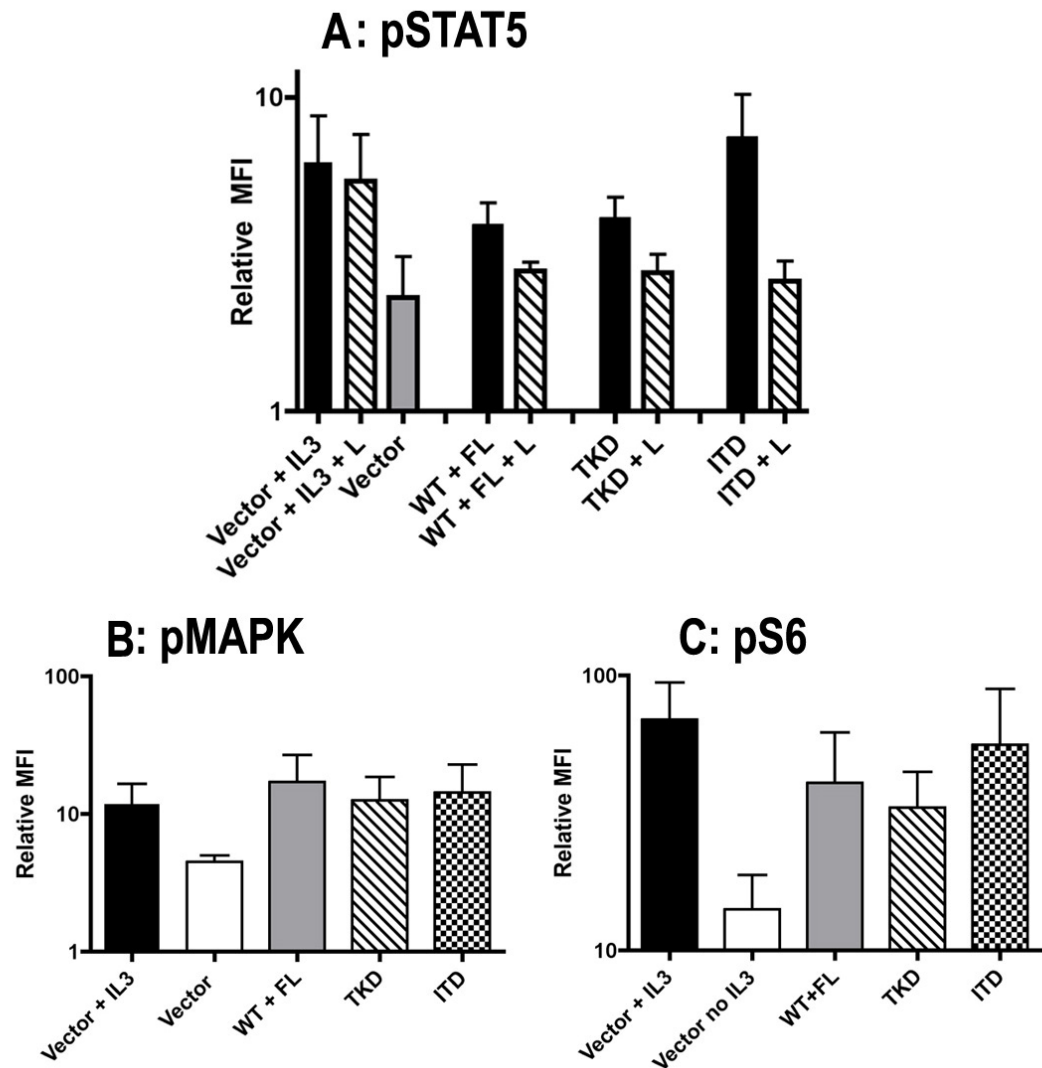


Figure 7.14: Results of 3 independent experiments on intracellular phospho-signalling proteins in transduced Ba/F3 cells. (A) phospho-STAT5; (B) phospho-MAPK; and (C) phospho-S6. The median fluorescence intensity (MFI) is expressed as a ratio of the MFI relative to control cells which were not incubated with the primary antibody. All y axes are on a logarithmic scale.

strong pSTAT5 fluorescence which was markedly reduced by pre-incubation with lestaurtinib.

Similar experiments were performed to assess the level of pMAPK and phospho-S6 (Figure 7.14B and C). Phospho-S6 was used as this is downstream of AKT, and therefore indicates AKT activation, as pAKT itself is difficult to detect by flow cytometry due to low levels of fluorescence intensity. No significant difference in the activation of pMAPK or phospho-S6 was seen between Ba/F3-vector cells grown in the

presence of 10 nM mIL3, Ba/F3-WT cells grown in the presence of 20 ng/ml FL or cytokine free, Ba/F3-TKD or Ba/F3-ITD cells. The impact of the addition of lestaurtinib was not tested in these experiments.

7.3.6 The impact of FLT3 expression on ATRA-induced differentiation of NB4 cells

To examine the impact of FLT3 mutants on haematopoietic cell differentiation, ATRA-induced expression of CD11b was examined in the promyelocytic leukaemia cell line, NB4. Incubation of NB4-vector cells with ATRA for four days led to an ATRA-concentration dependent increase in CD11b expression (Figure 7.15A, E). NB4-WT and NB4-TKD cells showed an increase in CD11b expression relative to NB4-vector cells at intermediate concentrations of ATRA (Figure 7.15 B, C, E). Conversely, NB4-ITD cells showed less CD11b expression at the lower ATRA concentrations (Figure 7.15D, E). This effect was overcome at a higher concentration of ATRA (1 μ M). By arbitrarily setting a gate at 2% in untreated cells, the relative proportion of NB4 cells that differentiated in response to ATRA could be quantified. In the experiment shown in Figure 7.15, 0.11 μ M ATRA induced differentiation in 66%, 76%, 84% and 53% of vector, WT, TKD and ITD-transduced cells respectively. Similar results were obtained in 3 independent experiments.

The difference between the 4 cell lines was most marked at 0.11 μ M ATRA. In order to determine whether the differences in CD11b expression were reproducible, 3 independent experiments were performed using 0.11 μ M ATRA. In addition, the impact of lestaurtinib on the level of ATRA-induced CD11b expression was examined. A concentration of 10 nM lestaurtinib was used as this had a minimal impact on cell viability in all NB4 lines. Figure 7.16A shows the mean MFI for each condition. Due to marked variation in the MFI between experiments, the SEMs are considerable and consequently the differences are not significant. Therefore, to compare the different FLT3-transduced cell lines, results were expressed as a ratio of CD11b MFI relative to that of NB4-vector cells for each condition (Figure 7.16B). In this analysis, NB4-TKD cells consistently showed increased CD11b expression in response to ATRA relative to NB4-vector cells whereas NB4-ITD cells showed reduced CD11b expression. Lestaurtinib inhibited the relative increase in ATRA-induced CD11b expression in NB4-TKD cells and increased CD11b expression in NB4-ITD cells. Therefore, in the

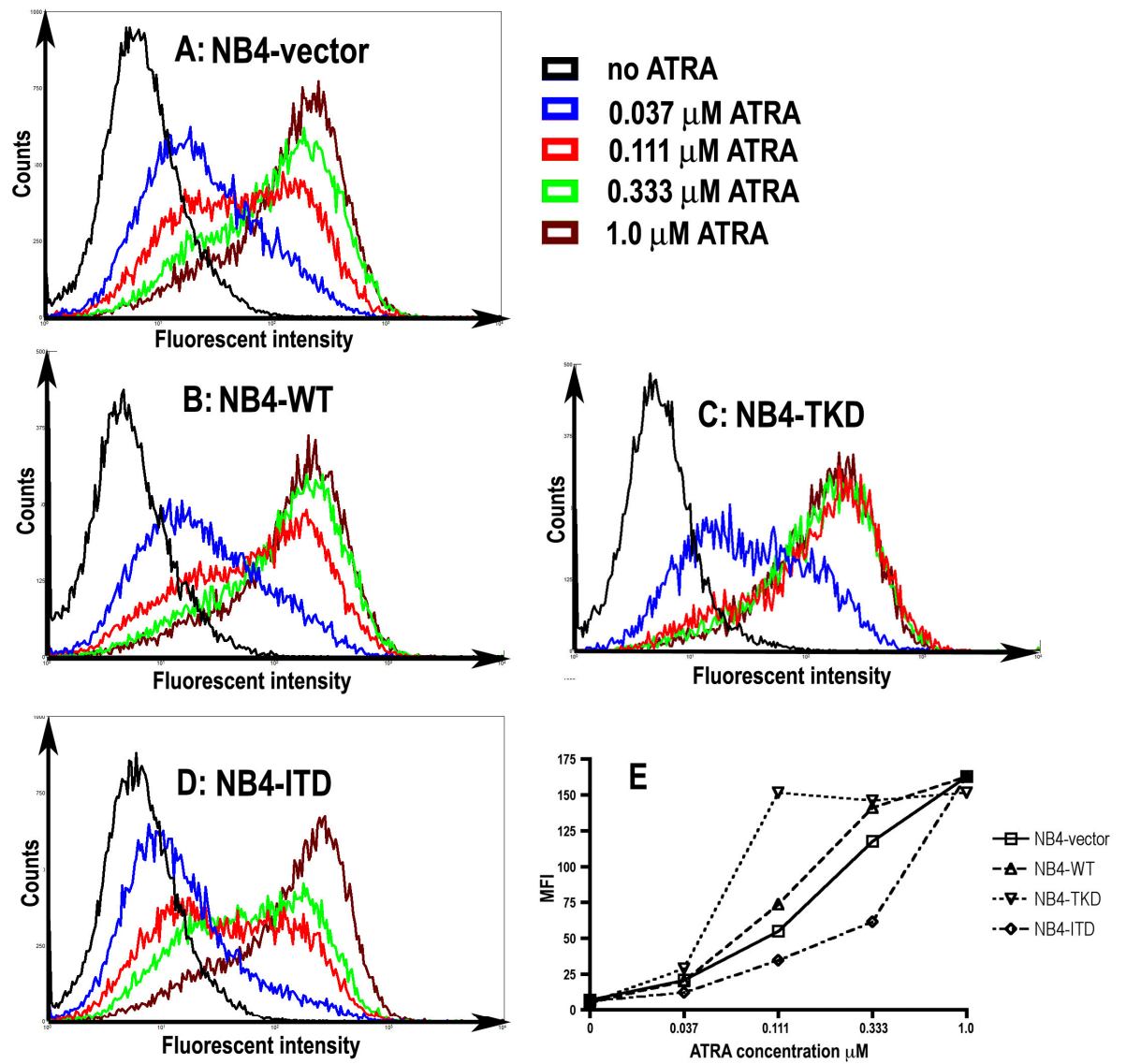


Figure 7.15: ATRA-induced CD11b expression in transduced NB4 cells. (A) NB4-vector; **(B)** NB4-WT; **(C)** NB4-TKD; **(D)** NB4-ITD; and **(E)** dose response relationship between ATRA concentration and median fluorescent intensity of CD11b.

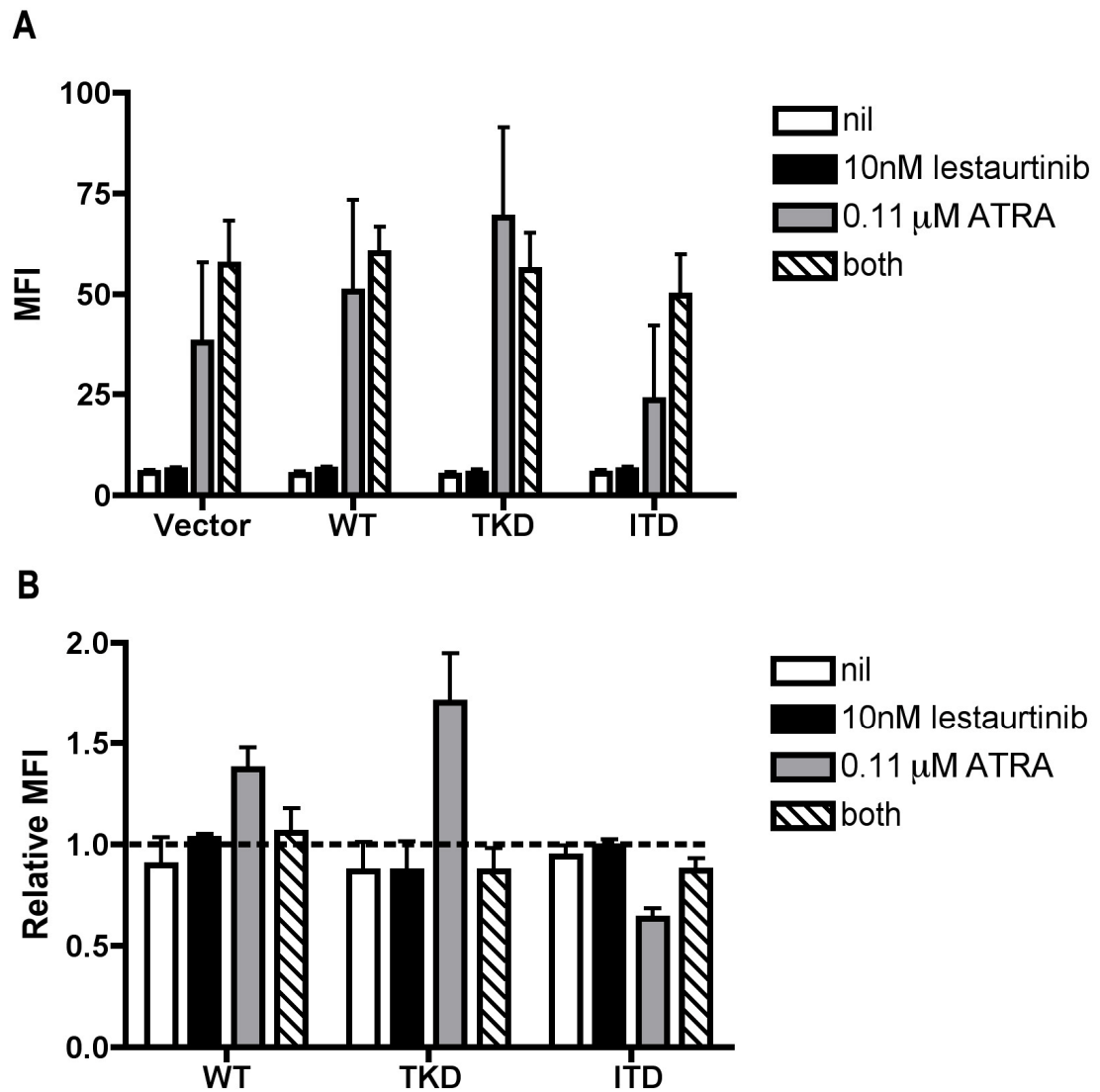


Figure 7.16: ATRA-induced CD11b expression in transduced NB4 cells and the impact of lestaurtinib. (A) Mean and SEM of MFI; (B) results expressed as the relative MFI compared to vector transduced cells. Data shown is the result of 3 independent experiments.

presence of lestaurtinib, all 4 cell lines responded similarly to ATRA in terms of CD11b expression, suggesting that the observed differences are FLT3- dependent.

7.3.7 Lentiviral transduction of primary CD34+ cells

In view of the limitations of transformed cell line models for the study of FLT3 mutations, a lentiviral vector system was developed for transduction of CD34+ haematopoietic stem cells. This required considerable optimisation before significant transduction efficiencies were achieved. The necessary titres of virus required for the

transductions were easily obtained for the 'empty vector', but viral production was far less efficient for the FLT3 constructs. Furthermore, the use of polybrene and/or retronectin was associated with considerable cell death in the FLT3-transduced CD34+ cells. Even following optimisation and with an MOI of 50, transduction efficiency for the FLT3-containing constructs was poor. In 3 independent experiments, for CD34-vector transductions the %GFP-positive cells at 72 hours were 29%, 40% and 49% respectively. For CD34-WT the transduction was only 6%, 17% and 8%, for CD34-TKD 10%, 11% and 5%, and for CD34-ITD 7%, 7% and 6% respectively.

In order to assess the impact of FLT3 expression on the survival and/or proliferation of CD34+ cells grown in cytokine-free liquid culture, following the initial 72 hours in cytokines, unsorted cells were washed and cultured in cytokine-free liquid culture. The total number of viable cells and the proportion that were GFP-positive were counted every 1-2 days over the course of a 2 week period using flow cytometry and propidium iodide staining with calibration beads as described in section 7.2.12. In the absence of cytokines, all CD34+ cells showed a rapid decline in viability, irrespective of the construct they had been transduced with, and there was no evidence, therefore, that mutant FLT3 led to factor-independent proliferation of CD34+ cells (Figure 7.17A). The percentage of GFP-positive cells, however, did increase over the two week period for all the FLT3-transduced cells (Figure 7.17B). For example, over 3 independent experiments, after 7 days of culture, the proportion of GFP+ cells increased from 10% to 30% for FLT3/WT transduced cells, 9% to 29% for FLT3/TKD cells and 7% to 25% for FLT3/ITD cells. However, the proportion of GFP-expressing cells also increased for CD34-vector cells from 39% to 49%, suggesting that these findings may, at least partly, relate to an increase in auto-fluorescence.

7.3.8 Colony assays of transduced CD34+ cells

Following 14 days in cytokine-free liquid culture, 250 unsorted CD34+ cells transduced with the different constructs were added to 0.5 mls of methycellulose in the presence of 20ng/ml SCF, 20ng/ml FL, 20 ng/ml GM-CSF and 50ng/ml G-CSF and placed in 5% CO₂ in a humidified incubator at 37°C. The number of colonies was counted after 2 weeks. Each colony assay was performed in triplicate and the results presented are the mean numbers of colonies from 3 independent experiments. Of the initial 2×10^5 transduced CD34+ cells, the estimated number of colony forming cells remaining after 2 weeks in cytokine-free liquid culture was 74 ± 24 for vector-transduced cells (Figure

7.17C). This was not different for FLT3/WT- (114 ± 35 , P.4) or TKD-transduced cells (124 ± 46 , P.4) but was markedly increased for FLT3/ITD-transduced cells (642 ± 222 , P.03).

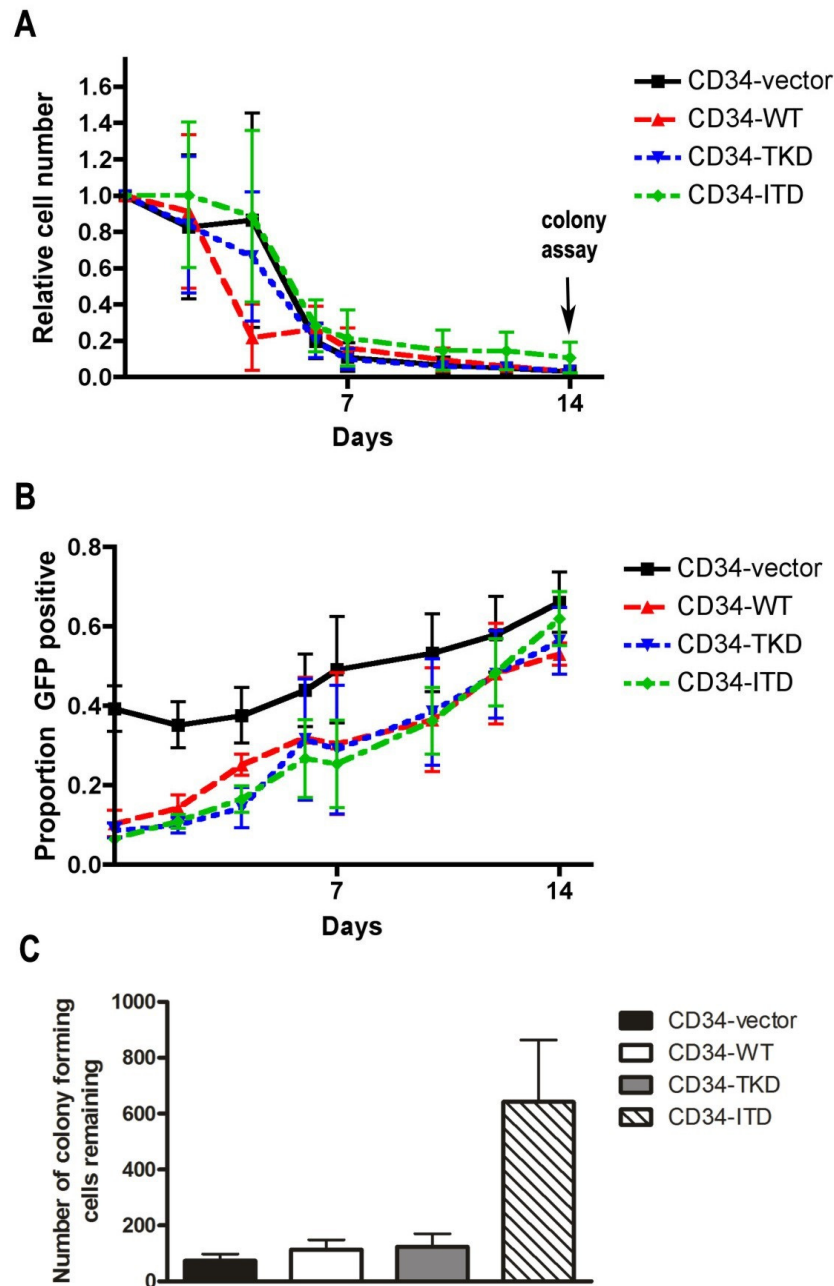


Figure 7.17: Transduction of CD34⁺ cells with vector or FLT3 constructs. (A) Relative numbers of viable transduced CD34 cells in cytokine free liquid culture; **(B)** Proportion of GFP expressing cells over 2 weeks in cytokine free liquid culture; and **(C)** numbers of colony forming units remaining after cytokine free liquid culture for 14 days.

7.4 Discussion

A number of studies of AML patients have observed clinical and biological differences between AML patients with FLT3/TKD mutations and FLT3/ITDs (reviewed in Chapters 4, 5 and 6). The data described in chapters 4, 5 and 6 adds to the accumulating evidence of important differences between the 2 mutations. It remains unclear, however, whether these differences directly relate to differential signalling events downstream of the mutations or to an association of these different mutation with other biological variables such as different cytogenetic abnormalities or mutations, in which case, the functional effects of the two mutations would be expected to be the same. In order to explore possible signalling differences between FLT3/TKD mutations and FLT3/ITDs, a number of cell model systems were developed whereby different FLT3 constructs were introduced into the 32Dcl3, Ba/F3, NB4 cell lines and primary human CD34+ peripheral blood mobilised cells. This allowed direct comparison in the same cellular context of the impact of the 2 mutations on cell survival, proliferation and differentiation, as well as *in vitro* sensitivity to therapeutic agents.

In the initial experiments, the impact of the expression of the different FLT3 mutations on the growth of 32Dcl3 and Ba/F3 cell lines was assessed. These cell lines are normally dependent on the presence of mIL3 for cell survival and proliferation. In the absence of mIL3, vector-transduced cells failed to proliferate and died within a few days (Figures 7.6 and 7.8). Expression of both the FLT3/ITD and FLT3/TKD mutation led to factor-independent growth of both cell lines. FLT3/ITD-transduced cells, however, proliferated at a more rapid rate than FLT3/TKD-transduced cells. This difference was unlikely to be due to quantitative differences in the amount of FLT3 protein present in the cells as they were selected according to the level of GFP expression, which should be proportional to FLT3 expression, and equivalent expression of FLT3 protein was confirmed by immunoblotting (Figure 7.5). Surface expression of FLT3 was not used to select cells with equivalent FLT3 expression as there is evidence that FLT3/ITDs are able to signal when located intracellularly as well as when at the cell surface (Koch *et al*, 2008). Furthermore, Koch *et al* also showed that overexpressed FLT3/WT accumulated within the endoplasmic reticulum of COS7 cells rather than at the cell surface. The present data, therefore, suggest that FLT3/ITDs confer a stronger proliferative drive, at least in the context of these murine cell model

systems. These results are consistent with previous reports (Choudhary *et al*, 2005b, Grundler *et al*, 2003).

Mutation of FLT3 is not the only possible mechanism of aberrant FLT3 signalling. For example, there are several lines of evidence that FLT3/WT protein is expressed at high levels in some cases of AML (Armstrong *et al*, 2003, Caligiuri *et al*, 2007, Dicker *et al*, 2007, Sargin *et al*, 2007, Zheng *et al*, 2004b). However, the biological significance of FLT3/WT overexpression is unclear as it has not been shown to lead to constitutive FLT3 signalling. Of note, the present data show that in both 32Dcl3 and Ba/F3 cells, expression of FLT3/WT alone was sufficient to lead to cell survival in the absence of cytokines, although these cells only proliferated at a very slow rate (Figures 7.6, 7.7 and 7.8). The addition of FL to these cells led to a marked increase in the rate of proliferation to a level close to that of FLT3/TKD-transduced cells. The observation that FLT3/WT overexpression, with or without FL, is capable of conferring a survival and proliferative signal is important as this may, therefore, contribute significantly to cell survival and proliferative signalling in AML, particularly if FL is co-expressed (Zheng *et al*, 2004b). This may also be important with regards to the potential use of FLT3 inhibitors in patients with FLT3/WT as discussed in Chapter 6.

In view of the marked impact of FL on the proliferation rate of FLT3/WT transduced cells, it is also possible that FL may influence the proliferative signals induced by FLT3 mutations. The impact of the addition of FL to 32cl3D-TKD and 32cl3D-ITD cells was therefore examined. FL only led to marginal increase in the proliferation rate of FLT3/TKD-transduced 32Dcl3 cells and was insufficient to induce a proliferation rate equivalent to that of FLT3/ITD transduced cells. The distinct proliferation signal from the two mutations is not, therefore, accounted for by a differential response to FL.

The dependence of cell proliferation on signalling through FLT3 in these model systems was supported by the impact of FLT3 inhibition. Lestaurtinib inhibited proliferation of FLT3/ITD- and FLT3/TKD-transduced 32Dcl3 and Ba/F3 cells and led to cell death. It is interesting, however, that TKD-transduced cells were more sensitive to the cytotoxic effects of lestaurtinib than ITD-transduced cells (Figure 7.10). This resulted from reduced cell viability and did not simply reflect a different rate of proliferation between TKD- and ITD-transduced cells (Figure 7.11). This cytotoxic effect was not abrogated by the addition of FL. These data are of particular interest in view of the opposite

observation in primary AML samples (Chapter 6) where FLT3/ITD-positive blast cells were more sensitive to the cytotoxic effects of lestaurtinib than FLT3/TKD mutant-positive blast cells. There are a number of possible explanations for these differences. First and foremost, primary cells are characterised by multiple genetic alterations that have been acquired over a prolonged period of time and they may not, therefore, be dependent on one signal for cell survival. Furthermore, as discussed in previous chapters, ITDs and TKD mutations may be associated with different genetic abnormalities that may also influence the response to FLT3 inhibition. Conversely, transduced factor-dependent cell line models are, by definition, dependent on the introduced oncogene for survival. Secondly, it may also be that the different FLT3/TKD mutations in the primary blast cell cases in Chapter 6 (only 3 of 11 were D835Y) are less sensitive to lestaurtinib than the D835Y mutation used in all the experiments described in this chapter. Alternatively, if FLT3/ITDs confer a stronger survival and proliferative signal than FLT3/TKDs, this may have different implications with regards to the sensitivity to the cytotoxic effects of lestaurtinib depending on the cellular context. In cell line models such as the 32Dcl3 and Ba/F3 lines used in this chapter, a lower concentration of lestaurtinib may be sufficient to reduce the weaker survival and proliferative signals induced by a FLT3/TKD to a level that is incompatible with cell survival. In contrast to this, the stronger survival and proliferative signals of FLT3/ITDs may require higher concentrations of lestaurtinib to lead to the necessary level of inhibition to induce cell death. Conversely, as discussed in Chapter 6, in primary cells, the stronger survival signals downstream from a FLT3/ITD may increase the dependence of the blast cells on this signal for survival. This may, therefore, increase sensitivity to the cytotoxic effects of lestaurtinib thus providing indirect evidence for ‘oncogene addiction’ to FLT3 signalling in AML (Weinstein and Joe 2008).

It is possible that the distinct proliferative impacts and differences in sensitivity to therapeutic agents between FLT3 mutations may relate to differential signalling events. Published studies are conflicting in this regard. For example, whilst a number of previous studies had shown that ITDs induce strong phosphorylation of STAT5A (Choudhary *et al*, 2005a, Choudhary *et al*, 2005b, Grundler *et al*, 2005), the impact of FLT3/TKD mutations and/or FL-stimulated FLT3/WT on STAT5A activation is controversial. Some studies have demonstrated that FLT3/TKD mutations (Grunder *et al*, 2003) and FLT3/WT (Zhang *et al*, 2000) may also strongly activate STAT5A. Others, however, have suggested that FLT3/WT and FLT3/TKDs fail to induce strong

STAT5A phosphorylation, both in cell lines (Choudhary *et al*, 2005b, Hayakawa *et al*, 2000, Mizuki *et al*, 2000) and an animal model (Grundler *et al*, 2005). Furthermore, one study also suggested that FLT3/TKDs less strongly activated ERK1 and 2 compared with FLT3/ITDs (Choudhary *et al*, 2005b).

The transduced Ba/F3 cell lines described in this chapter also provided an opportunity to examine the downstream signalling events that occur as a result of the presence of different types of FLT3 mutation. Three different signalling pathways, MAPK, PI3K and STAT5 were investigated using immunoblotting of cell lysates and intracellular flow cytometry of single cells. The study of intracellular signalling events in fixed and permeabilised single cells by flow cytometry was a useful technique for these studies as it allowed a degree of quantification of signalling differences. A clear difference in the degree of activation of STAT5A was observed between Ba/F3-ITDs, which showed strong activation of STAT5A, and Ba/F3-TKD or FL-stimulated Ba/F3-WT cells, which only demonstrated weak STAT5A activation (Figures 7.12 and 7.13). STAT5A activation in mIL3-stimulated Ba/F3-vector cells was not affected by the addition of the FLT3 inhibitor, whereas in the FLT3-ITD and TKD-transduced Ba/F3 cells, STAT5A activation was fully inhibited by the addition of lestaurtinib. PI3K and MAPK pathway activation appeared to be comparable between the two types of mutated FLT3, although smaller differences cannot be excluded. Further studies will be required to determine whether differential STAT5 activation accounts for the proliferative differences between FLT3/ITDs and TKD mutations.

In addition to aberrant cell survival and/or growth, a block in cellular differentiation is also a feature of a full leukaemic phenotype. This is often simplified as a two-hit model of leukaemogenesis, as discussed in Chapter 1, whereby a type-1 mutation confers a proliferative and survival drive and a type-2 mutation leads to a differentiation block (Kelly and Gilliland 2002). However, a number of different genetic events can lead to both type-1 and type-2 effects. For example, in limited *in vitro* studies, FLT3/ITDs have been shown to lead to a differentiation block in haemopoietic cell line models (Schwable *et al*, 2005, Zheng *et al*, 2002). The impact of FLT3/TKD mutations on differentiation has not been investigated. A model system of ATRA-induced CD11b expression was therefore developed in FLT3/WT and FLT3 mutant NB4 cells. As anticipated from previous studies, these data demonstrated that transduction of NB4 cells with a FLT3/ITD led to reduced ATRA-induced differentiation at lower

concentrations of ATRA compared to vector transduced cells (Figures 7.15 and 7.16). In contrast, NB4-WT and NB4-TKD cells demonstrated increased differentiation in response to ATRA. This was not related to a difference in the rate of proliferation, which was identical between the different transduced cell lines (Figure 7.9). These data suggest that there may be important differences in the downstream effects of the different mutations on haematopoietic transcription factor expression levels that control cellular differentiation. It is interesting to note, therefore, that studies have shown FLT3/TKDs, unlike ITDs, do not suppress C/EBP α or PU.1 expression (Choudhary *et al*, 2005b), two transcription factors that are key regulators of myeloid cell differentiation. FLT3/TKDs also lead to an increased expression of the tumour suppressor TSC-22, which in turn promotes, rather than blocks, myeloid differentiation (Lu *et al*, 2007). The impact of FLT3/ITDs on TSC-22 expression is not known. This differential impact of ITDs and TKD mutations on haematopoietic cell differentiation is interesting in view of the observations described in Chapter 4 that FLT3/TKD mutations and FLT3/ITDs show markedly different associations with other genetic abnormalities. For example, FLT3/TKDs are common in cases with the inv(16) cytogenetic abnormality whereas FLT3/ITDs are uncommon in this subgroup. This may reflect the distinct impact of different FLT3 mutations on haematopoietic cell differentiation and therefore their ability to collaborate with other genetic events.

Studies of the impact of FLT3 mutations in immortalised and transformed cell lines can provide useful information on signalling events but such cell lines are far removed from the impact of FLT3 mutations in primary AML cells *in vivo*. Therefore, in order to study the impact of FLT3 mutations on primary human haematopoietic progenitor cells, a lentiviral system for CD34⁺ cell transduction was developed. However, despite numerous attempts at optimisation, transduction efficiencies were low. Nevertheless, transduction was sufficient to allow some observations. Firstly, neither the presence of a FLT3/ITD nor a FLT3/TKD mutation led to factor-independent proliferation of CD34⁺ cells in cytokine-free liquid culture. Previous studies had shown that expression of FLT3/ITD in CD34⁺ cells leads to a proliferative advantage over WT-transduced cells using stromal co-culture assays, suggesting that additional signals are required in primary cells in addition to those downstream of a FLT3/ITD (Chung *et al*, 2005, Moore *et al*, 2007). Another study had shown that in a liquid culture system of FLT3-transduced cells, further cytokines are required such as thrombopoietin and SCF in addition to the FLT3 signalling in order to allow cell survival and proliferation (Li *et al*,

2007). Despite the lack of factor-independent proliferation in the absence of other cytokines in the present studies, expression of FLT3 did lead to an increase in the percentage of GFP-positive cells with time, although this did not differ between FLT3/WT, TKD and ITD-expressing cells. These findings should be interpreted with caution, however, in view of the increase in the percentage of GFP-expressing vector-transduced cells, as they may simply relate to an increase in autofluorescence with time, regardless of the presence of a FLT3 mutation. The expression of a FLT3/ITD did lead to a significant increase in the number of colony forming units that were able to survive for 1 or 2 weeks in cytokine-free liquid culture. It is possible that the different effects of FLT3/ITD and FLT3/TKD mutations on cell survival, proliferation and differentiation may explain these observations. However, in view of the poor transduction efficiency in the experiments described in this chapter, a more reliable system for transduction of human CD34⁺ cells will be required in order to allow more systematic study of the impact of FLT3 mutations in primary human cells.

In conclusion, the data presented in this chapter provides a number of lines of evidence in multiple cell line and primary cell model systems that FLT3/ITDs lead to a stronger survival and proliferative signal than occurs as a result of a FLT3/TKD. In these studies, FLT3/TKDs confer similar signalling as that of FL-stimulated FLT3/WT cells whereas FLT3/ITD signalling was more akin to that of IL3 stimulated cells. These studies also confirmed previous observations that FLT3/ITDs induce a myeloid differentiation block. Conversely, however, FLT3/TKDs that appeared to promote haematopoietic cell differentiation. Whether these differences occur as a result of the observed differential activation of STAT5A by the two mutations remains to be established. Furthermore, exactly how these observations relate to the clinical differences observed between FLT3/ITDs and FLT3/TKD mutations remains unclear.

CHAPTER 8: CONCLUSIONS AND FUTURE DIRECTIONS

Despite significant improvements in the treatment of young adults with AML over the last few decades, outcome is still unsatisfactory with only approximately 40-50% of patients alive and disease-free after 5 years. As described in Chapter 1, risk stratification according to cytogenetics and clinical factors allows a degree of risk-adapted therapy in current protocols, enabling selection of higher risk patients for more intensive therapy. However, these prognostic groups include patients with markedly heterogeneous diseases and consequently, in many cases, useful information to guide treatment is not available. Improved understanding of the role of molecular abnormalities in AML may allow further dissection of these risk groups and more accurate risk stratification. Furthermore, this may also provide insights into the biology of AML and improve therapeutic strategies in the future. Whilst the presence of a FLT3/ITD is known to predict adverse outcome in young adults with AML, the clinical significance of FLT3/TKD mutations is unclear. In order to address this issue, the studies described in this thesis compared and contrasted the incidence (Chapter 3) and clinical associations (Chapters 4 and 5) of FLT3/TKD mutations with those of FLT3/ITDs in a large cohort of young adult patients with AML treated on the MRC AML 10 and 12 trials. The impact of FLT3 inhibitors according to FLT3 mutations status (Chapter 6) and the biological impact of different FLT3 mutations *in vitro* (Chapter 7) was also assessed. In the present Chapter, the main findings of these studies will be briefly summarised and possible directions for future studies highlighted.

The dHPLC-based method for the detection of FLT3/TKD mutations (Chapter 3) was shown to be a highly sensitive technique, capable of detecting FLT3/TKD mutations present in as few as 5% of FLT3 alleles. This technique was also shown to be superior to the commonly used *EcoRV* restriction digest technique as evidenced by 17 cases (11% of all mutant-positive cases) that were mutant by dHPLC but WT by *EcoRV* digestion. The dHPLC method also had the advantage that the chromatogram trace readily allowed identification of the type of mutation (Figure 3.5). Therefore, a cohort of 1339 young adults with AML from the UK MRC AML trials was screened for FLT3/TKD mutations using this dHPLC technique. Mutations were detected in 161 cases (12%).

There were a number of significant differences observed when the clinical characteristics and outcome of these FLT3/TKD mutant-positive cases was compared with FLT3/ITD positive cases (Chapter 4). For example, there was a high incidence of FLT3/TKD mutations in patients with inv(16) (13 of 55 patients, 24%, $P=.009$), a cytogenetic group in which FLT3/ITDs are uncommon. Strikingly, the clinical outcome of patients with FLT3/TKD mutations and FLT3/ITDs was markedly different, with FLT3/TKD mutations associated with a favourable long-term outcome in contrast with the adverse outcome associated with FLT3/ITDs. For example, the CIR at 10 years was 46% for FLT3 WT patients, 37% for FLT3/ITD⁻TKD⁺ patients, 62% for FLT3/ITD⁺TKD⁻ patients and 68% for FLT3/ITD⁺TKD⁺ patients. OS for these groups was 36%, 51%, 24% and 37% respectively ($P<.001$ for FLT3/ITD⁻TKD⁺ versus FLT3/ITD⁺TKD⁻ patients).

FLT3/TKD mutation screening by dHPLC also revealed marked variability in the amount of heteroduplex that was present in FLT3/TKD⁺ cases, most likely reflecting variability of FLT3/TKD mutant levels. Furthermore, analysis of FLT3/ITD mutations has shown that the relative level of mutant and WT alleles varies widely in AML and correlates with clinical characteristics and outcome (Gale *et al*, 2007). Therefore, the relative mutant level in FLT3/TKD⁺ cases was quantified (Chapter 5). The distribution of mutant levels was different to that of FLT3/ITDs. Cases with a very high level of mutation (>50% of total FLT3 alleles) were less common for FLT3/TKD mutations (9%) than for FLT3/ITDs (approximately 15%). Furthermore, only 2 (2%) cases had FLT3/TKD mutant levels greater than 75% compared with 8% for FLT3/ITDs. Conversely, low level mutants ($\leq 25\%$ of total FLT3 alleles) were more frequent for FLT3/TKDs, accounting for approximately 50% of mutant-positive patients compared with 29% of FLT3/ITDs. There was also a highly significant inverse relationship between the presence of high level FLT3/TKD and FLT3/ITD mutations, which never occurred together ($P<.001$).

Stratification of the cohort according to FLT3/TKD mutant level and clinical characteristics at presentation showed that high level mutants (>25% total FLT3 alleles) were associated with a high WBC at presentation, whereas low level mutants had a WBC that was similar to that of FLT3/WT patients. The favourable outcome of FLT3/TKD mutants described in Chapter 4 was mostly attributable to patients with a high level of mutant. Lower level mutant-positive patients had an outcome that was

very similar to that of FLT3/WT patients. For example, patients with a high level mutant had an OS at 10 years of 59% compared to 37% in patients with a lower level mutant and 33% in patients without a FLT3/TKD mutation. This difference remained significant in multivariate analysis ($P=.004$).

The marked differences observed between FLT3/TKD mutations and FLT3/ITDs raised the possibility that the *in vitro* and *in vivo* effects of FLT3 inhibitors may also differ according to the mechanism of FLT3 activation. The *in vitro* cytotoxic impact of lestaurtinib, a FLT3 inhibitor currently in phase III clinical trials, was therefore assessed (Chapter 6). Primary leukaemic blast cells from FLT3/ITD-positive patients were more sensitive to the cytotoxic effects of lestaurtinib than cells with a FLT3/TKD mutation. Interestingly, patients with a very high level FLT3/ITD mutation, associated with a particularly adverse prognosis, were particularly sensitive to lestaurtinib induced cytotoxicity. There was, however, no difference in the sensitivity of cells to cytarabine when stratified according to FLT3 mutation status. The concurrent incubation of lestaurtinib with cytarabine led to synergistic cytotoxicity. This synergistic cytotoxic effect did not differ when blast cells were incubated with cytarabine and lestaurtinib concurrently or sequentially.

In order to explore the possibility that the clinical differences between FLT3/TKD mutations and FLT3/ITDs relate directly to differential signalling events, a number of *in vitro* cell model systems were developed (Chapter 7). Different FLT3 constructs were introduced into 32Dcl3, Ba/F3, NB4 cell lines and primary human CD34+ peripheral blood mobilised cells. In 32Dcl3 and Ba/F3 cells, a FLT3/ITD led to a stronger proliferative drive than a FLT3/TKD mutation. In an NB4 cell line model of ATRA-induced myeloid differentiation, a FLT3/ITD inhibited differentiation unlike a FLT3/TKD mutation which increased differentiation. Furthermore, transduction of human, CD34+ HSCs with a FLT3/ITD led to increased cytokine-free survival of colony forming cells, unlike a FLT3/TKD which did not differ from WT. Signalling studies also revealed differences, with a FLT3/ITD leading to stronger STAT5 activation than a FLT3/TKD mutation or FL stimulation of FLT3/WT. In summary, the functional impact of a FLT3/TKD mutation was similar to that of FL-stimulated FLT3/WT cells whereas FLT3/ITD signalling was more akin to that of IL3-stimulated cells.

8.1 Future directions

The incidence of FLT3/TKD mutations that were detected in the present cohort was higher than has been described in most other studies examining different cohorts. Whilst it is possible that this was due to the increased sensitivity of the dHPLC technique, as well as the detection of mutations that occur outside the *EcoRV* digest site, it is also possible that this difference relates to a high proportion of cases of AML with a high presenting WBC that are stored in the MRC AML gDNA bank, hence biasing towards inclusion of FLT3/TKD mutant-positive patients. Ongoing studies of AML15 samples, using the dHPLC technique will address this question as samples are obtained from a very high proportion of all cases and analysis of these samples will, therefore, not be subject to a similar bias. Indeed, preliminary results suggest that the incidence of FLT3/TKD mutations is lower in these samples.

Whilst the difference in prognosis between FLT3/ITD and FLT3/TKD mutant-positive patients was statistically highly significant, the favourable prognosis of FLT3/TKD mutant-positive cases of AML in comparison with FLT3/WT cases requires further confirmation, as very different observations have been reported for other cohorts (Chapter 4). Whilst it is possible that differences between the present and other studies could be partly due to the different screening techniques used, it seems unlikely that this could wholly account for the contrasting results. An important factor relating to these differences may be heterogeneity of patient cohorts studied with regards to inclusion criteria, and also treatment protocols. For example, it is noteworthy that cases with a favourable *inv*(16) karyotype were over-represented in the FLT3/TKD mutant-positive group in the present cohort, although this was also the case in some, but not all, the other cohorts (Chapter 4). However, despite this, when stratified into different cytogenetic categories, the favourable prognosis of FLT3/TKD mutations remained statistically significant within the intermediate cytogenetic category (Figure 4.2B), although, in multivariate analysis the favourable prognosis was only of borderline statistical significance. It is also likely that other molecular abnormalities will also influence the clinical outcome of the patients studied, particularly within this crucial intermediate cytogenetic category, and information on some known interacting molecular abnormalities was not available in this analysis. For example, NPM1 and CEBPA mutations are frequent in NK patients, are known to coexist with both FLT3/TKD mutations and FLT3/ITDs and, importantly, are both associated with a

favourable prognosis in AML. It will be important, therefore, to re-analyse the FLT3/TKD mutation data when information with regards to these molecular abnormalities becomes available. It would be particularly interesting to examine a population of NK-AML patients with an NPM1 mutation stratified according to FLT3 mutation status. Within these groups of patients, the impact of the different FLT3 mutations could be studied in the absence of a number of some of the other confounding variables.

The lack of an adverse outcome associated with FLT3/TKD mutations is of direct clinical relevance as some centres are selecting patients for allogeneic transplantation in first remission on the basis of the presence of a FLT3/ITD (Litzow 2005). In the absence of other poor prognostic factors, this might not be appropriate in patients with FLT3/TKDs. The finding of a markedly different clinical outcome associated with activating mutations of the same gene also has broader implications for molecular screening as these data would suggest that mutations of the same gene should only be grouped together with caution and, where possible, after heterogeneity in clinical and biological characteristics has been addressed. Dissecting out the specific clinical associations of each type of mutation is challenging, particularly in view of the relatively low incidence of many mutations as well as the increasing number of different mutations being reported. However, the risk of misinterpreting data and consequently inappropriately treating patients on the basis of these results must also be taken into account.

Assessment of the relative mutant level as described in Chapter 5 not only provides additional clinically relevant information but also can further assist in understanding the role of these mutations in AML biology. With the advent of genome-wide approaches to mutation detection using next generation sequencing technology (Ley *et al*, 2008), it will become increasingly important to dissect mutations that are clinically and/or biologically 'important' from other mutations. However, determining approaches that allow the identification of the 'important' mutations is not straightforward. For example, mutations may occur in cancer cells as primary events i.e. essential for cancer cell propagation, or as secondary events that may contribute to disease phenotype at a later stage in oncogenesis. Furthermore, it has been suggested that some mutations may be 'passenger' rather than 'driver' events that do not contribute significantly to pathogenesis of the cancer but are incidentally selected for. It should be noted, however,

that this does not necessitate that these ‘passenger’ events are biologically insignificant, although this has been used as a strategy to differentiate ‘passenger’ from ‘driver’ mutations (Frohling *et al*, 2007). Furthermore, the degree to which the concepts of ‘primary versus secondary’ and ‘passenger versus driver’ mutations overlap is not clear. Measurement of mutant level can contribute significantly to these issues. For example, as discussed in Chapter 6, higher mutant levels are likely to correlate with mutations that are acquired as earlier events in leukaemogenesis. Conversely, low level mutations are likely to be present in only a subclone of the leukaemic cells, and therefore a secondary event. The data presented in Chapter 6, therefore, would suggest that FLT3/TKDs occur more frequently as late or secondary events compared with FLT3/ITDs. Moreover, although FLT3/ITD and FLT3/TKD ‘dual mutant’ cases can be detected, at a biological level these data would suggest that the two different mutations are unlikely to both occur as early events.

Whether mutant level is helpful in the differentiation of driver versus passenger mutations is less clear. On the one hand it could be argued that passenger mutations are likely to be high level if they have incidentally occurred in the cell that initially undergoes transformation to leukaemia. Alternatively, if leukaemic cells are gradually acquiring secondary events at an increasing rate over time due to increasing genomic instability, the occurrence of passenger events will also increase. It is, however, difficult to design studies to directly address these issues. Whilst it would be biologically interesting to observe the natural history of low level mutations over time, as most young patients are treated with cytotoxic agents at diagnosis, such samples are not available. An alternative would be to study the dynamics of engraftment of these low-level mutants following xenotransplantation. It may be that some, or even the majority, of the low-level FLT3/TKD mutations would disappear from the leukaemic clone altogether, having occurred as an incidental event in a leukaemic cell without long-term self-renewal properties. The presentation/relapse studies (Chapter 5), where all low level mutations were lost at relapse, would be consistent with this hypothesis. A related study would be to sort leukaemic cells according to the surface expression of different antigens and examine the level of mutant within different BM stem cell and progenitor subpopulations, e.g. according to CD34, CD38 and FLT3 expression. Determining the populations of cells within the BM that contain the mutations, and also those that do not, may provide new therapeutic opportunities whereby the LSC could be targeted leaving the HSC intact.

As the study of mutant level can provide valuable information in addition to mutant detection alone, it could be proposed that an assessment of the mutant level is important for all mutation detection in cancer, although a prerequisite for this kind of analysis is that non-malignant cells only constitute a small proportion of the sample. Furthermore, ‘cut-off’ mutant levels would need to be standardised before this information could be incorporated into clinical protocols. Whether the benefit of such analyses outweighs the costs incurred by the additional complexity of analysis is likely to differ depending on the context.

The differential sensitivity of different FLT3 mutations to lestaurtinib (Chapter 6) is of relevance to ongoing clinical studies using FLT3 inhibitors in patients with FLT3-mutated AML. These data suggest that FLT3/TKD mutant-positive patients should not be differentiated from those with FLT3/WT and both groups may benefit from FLT3 inhibitor therapy. Despite this, many trials currently exclude FLT3/WT patients. Clearly, however, the most important issue is the clinical outcome of patients treated with FLT3 inhibitors. With a number of large phase III studies currently underway, this question should be addressed in the near future. The most effective sequence for administration of FLT3 inhibitors in combination with cytotoxic chemotherapeutic agents also remains unclear. A widely held view is that the two different classes of agent should be used in sequence rather than concurrently, mainly due to results obtained from cell line studies (see Chapter 6). This is due to the concern that FLT3 may be driving proliferation and, therefore, inhibition of FLT3 will reduce the proliferation rate of leukaemic blast cells and hence reduce the cytotoxic impact of cell cycle-dependent agents. However, the crucial role of FLT3 inhibitors may actually be to inhibit anti-apoptotic signalling in the LSC population. These LSCs are thought to be relatively resistant to cell cycle-specific agents such as cytarabine due to an inherent low rate of proliferation, paralleling their normal HSC counterparts. If this were the case then concurrent administration of FLT3 inhibitors with chemotherapy would be the most sensible approach, as the benefit of inhibition of anti-apoptotic signalling would outweigh the risk of inhibiting cell proliferation in an already relatively quiescent cell. This issue is worthy of further investigation in appropriate animal models in order to maximise the impact of anti-FLT3 therapies against the LSC population.

Chapter 7 explored the functional consequences of the different types of FLT3 mutation. It is possible that the increased proliferative drive and survival signalling that were associated with a FLT3/ITD directly relate to the increased chemoresistance observed in the FLT3/ITD-positive patient group. In addition to this, the increased differentiation of NB4 cells transduced with a FLT3/TKD mutation in contrast to impaired differentiation induced by expression of a FLT3/ITD was a novel finding. In the light of this, it would be interesting to explore the impact of ATRA on FLT3/TKD mutant-positive patients with AML who received this agent as part of the MRC trials as it is possible that they may selectively benefit from this therapy. Whether the differences between FLT3/ITDs and FLT3/TKD mutations directly related to the differential activation of STAT5, however, was not directly addressed in these studies. As STAT5 activation has been shown to be associated with impaired myeloid differentiation (Wierenga *et al*, 2006), it would be interesting to explore this further, for example, by studying the impact of FLT3 mutations on a STAT5A null background.

It should be noted, however, that viral transduction of cell-lines creates a highly artificial system that is, in many ways, far removed from AML pathogenesis. In an attempt to partly address these limitations, primary human haematopoietic cells were lentivirally transduced with different FLT3 constructs. However, the low transduction efficiency precluded more detailed study of these transduced cells beyond the described cytokine-free survival studies. This low transduction efficiency may reflect toxicity induced by unregulated expression of an activated oncogene in primary human cells. It may be, for instance, that the mutations causing AML are required to occur in a particular sequence with, for example, an NPM1 mutation being permissive for a cell to tolerate the presence of activated FLT3. This is clearly speculative, and in order to dissect these, and other, pathogenic mechanisms in AML, it will be essential to develop models of AML that more closely recapitulate the role of different mutations, including FLT3 mutations, in AML.

Viral transduction of murine haematopoietic cells followed by transplantation into recipient mice is a common strategy for the study of the impact of different mutations *in vivo*. It is important, however, to understand the limitations of this approach. First, viral transduction studies are complicated by the very high level of expression of the transgene which may not reflect the pathophysiological situation. As over-expression of FLT3/WT may alone contribute to leukaemogenesis, this is clearly of importance.

Second, insertional mutagenesis may contribute to virally transduced murine models of FLT3 mutations with unpredictable consequences induced by insertion of the viral promoter in close proximity to other oncogenes. Third, as in the majority of cases the whole BM compartment is transduced using these approaches, they do not contribute to understanding the possible target cell of transformation. Conditional knock-in of FLT3 mutations at their physiological locus, for example using a Cre-Lox approach, would go some way to addressing a number of these pitfalls. As it is essential to understand how mutations collaborate, the sequential or concurrent introduction of collaborating mutations is also important to study, perhaps using a combination of conditional knock-in of one mutation followed by viral transduction of another. With regards to FLT3/TKD mutations, NPM1 mutations and inv(16) are the most common, known collaborating genetic events, and are worthy of further study. Finally, it is possible that murine cells will respond to mutations in different ways to human cells. As it has now been shown to be possible to recapitulate human leukaemia using viral transduction strategies (Barabe *et al*, 2007), it would be interesting to try to reproduce these findings using a combination of different collaborating mutations, e.g. a FLT3 mutation and an NPM1 mutation. This would thereby provide a powerful model for the study of AML biology and therapy.

8.2 Conclusions

In conclusion, this thesis has described the distinct clinical and biological characteristics of FLT3/TKD mutations in AML in comparison with FLT3/ITDs. These findings not only provide a number of novel avenues for future studies but are also of direct clinical relevance to current clinical decision making in patients with AML. As such this work represents a significant contribution to current understanding of the role of FLT3 mutations in AML.

REFERENCES

- Abu-Duhier, F.M., Goodeve, A.C., Wilson, G.A., Care, R.S., Peake, I.R. & Reilly, J.T. (2001) Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia. *Br J Haematol*, **113**, 983-988.
- Abu-Duhier, F.M., Goodeve, A.C., Wilson, G.A., Gari, M.A., Peake, I.R., Rees, D.C., Vandenberghe, E.A., Winship, P.R. & Reilly, J.T. (2000) FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. *Br J Haematol*, **111**, 190-195.
- Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmiter, R.D. & Brinster, R.L. (1985) The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature*, **318**, 533-538.
- Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A., Anderson, K., Sitnicka, E., Sasaki, Y., Sigvardsson, M. & Jacobsen, S.E. (2005) Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell*, **121**, 295-306.
- Andersson, A., Johansson, B., Lassen, C., Mitelman, F., Billstrom, R. & Fioretos, T. (2004) Clinical impact of internal tandem duplications and activating point mutations in FLT3 in acute myeloid leukemia in elderly patients. *Eur J Haematol*, **72**, 307-313.
- Arland, M., Fiedler, W., Samalecos, A. & Hossfeld, D.K. (1994) Absence of point mutations in a functionally important part of the extracellular domain of the c-kit proto-oncogene in a series of patients with acute myeloid leukemia (AML). *Leukemia*, **8**, 498-501.
- Armstrong, S.A., Kung, A.L., Mabon, M.E., Silverman, L.B., Stam, R.W., Den Boer, M.L., Pieters, R., Kersey, J.H., Sallan, S.E., Fletcher, J.A., Golub, T.R., Griffin, J.D. & Korsmeyer, S.J. (2003) Inhibition of FLT3 in MLL. Validation of a therapeutic target identified by gene expression based classification. *Cancer Cell*, **3**, 173-183.
- Au, W.Y., Fung, A.T., Ma, E.S., Liang, R.H. & Kwong, Y.L. (2004) Low frequency of FLT3 gene internal tandem duplication and activating loop mutation in therapy-related acute myelocytic leukemia and myelodysplastic syndrome. *Cancer Genet Cytogenet*, **149**, 169-172.
- Auewarakul, C.U., Sritana, N., Limwongse, C., Thongnoppakhun, W. & Yenchitsomanus, P.T. (2005) Mutations of the FLT3 gene in adult acute myeloid leukemia: determination of incidence and identification of a novel mutation in a Thai population. *Cancer Genet Cytogenet*, **162**, 127-134.
- Bacher, U., Haferlach, C., Kern, W., Haferlach, T. & Schnittger, S. (2008) Prognostic relevance of FLT3-TKD mutations in AML: the combination matters - an analysis of 3082 patients. *Blood*, **111**, 2527-2537.
- Baldus, C.D., Tanner, S.M., Ruppert, A.S., Whitman, S.P., Archer, K.J., Marcucci, G., Caligiuri, M.A., Carroll, A.J., Vardiman, J.W., Powell, B.L., Allen, S.L., Moore, J.O., Larson, R.A., Kolitz, J.E., de la Chapelle, A. & Bloomfield, C.D. (2003) BAALC expression predicts clinical outcome of de novo acute myeloid leukemia patients with normal cytogenetics: a Cancer and Leukemia Group B Study. *Blood*, **102**, 1613-1618.
- Baldus, C.D., Thiede, C., Soucek, S., Bloomfield, C.D., Thiel, E. & Ehninger, G. (2006) BAALC expression and FLT3 internal tandem duplication mutations in acute

- myeloid leukemia patients with normal cytogenetics: prognostic implications. *J Clin Oncol*, **24**, 790-797.
- Barabe, F., Kennedy, J.A., Hope, K.J. & Dick, J.E. (2007) Modeling the initiation and progression of human acute leukemia in mice. *Science*, **316**, 600-604.
- Barjesteh van Waalwijk van Doorn-Khosrovani, S., Erpelinck, C., van Putten, W.L., Valk, P.J., van der Poel-van de Luytgaarde, S., Hack, R., Slater, R., Smit, E.M., Beverloo, H.B., Verhoef, G., Verdonck, L.F., Ossenkoppele, G.J., Sonneveld, P., de Greef, G.E., Lowenberg, B. & Delwel, R. (2003) High EVI1 expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. *Blood*, **101**, 837-845.
- Barragan, E., Cervera, J., Bolufer, P., Ballester, S., Martin, G., Fernandez, P., Collado, R., Sayas, M.J. & Sanz, M.A. (2004) Prognostic implications of Wilms' tumor gene (WT1) expression in patients with de novo acute myeloid leukemia. *Haematologica*, **89**, 926-933.
- Basecke, J., Whelan, J.T., Griesinger, F. & Bertrand, F.E. (2006) The MLL partial tandem duplication in acute myeloid leukaemia. *Br J Haematol*, **135**, 438-449.
- Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A., Gralnick, H.R. & Sultan, C. (1976) Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol*, **33**, 451-458.
- Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A., Gralnick, H.R. & Sultan, C. (1985) Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med*, **103**, 620-625.
- Bergmann, L., Maurer, U. & Weidmann, E. (1997) Wilms tumor gene expression in acute myeloid leukemias. *Leuk Lymphoma*, **25**, 435-443.
- Bianchini, M., Ottaviani, E., Grafone, T., Giannini, B., Soverini, S., Terragna, C., Amabile, M., Piccaluga, P.P., Malagola, M., Rondoni, M., Bosi, C., Baccarani, M. & Martinelli, G. (2003) Rapid detection of Flt3 mutations in acute myeloid leukemia patients by denaturing HPLC. *Clin Chem*, **49**, 1642-1650.
- Boissel, N., Cayuela, J.M., Preudhomme, C., Thomas, X., Grardel, N., Fund, X., Tigaud, I., Raffoux, E., Rousselot, P., Sigaux, F., Degos, L., Castaigne, S., Fenaux, P. & Dombret, H. (2002) Prognostic significance of FLT3 internal tandem repeat in patients with de novo acute myeloid leukemia treated with reinforced courses of chemotherapy. *Leukemia*, **16**, 1699-1704.
- Bonnet, D. & Dick, J.E. (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*, **3**, 730-737.
- Bos, J.L., Toksoz, D., Marshall, C.J., Verlaan-de Vries, M., Veeneman, G.H., van der Eb, A.J., van Boom, J.H., Janssen, J.W. & Steenvoorden, A.C. (1985) Amino-acid substitutions at codon 13 of the N-ras oncogene in human acute myeloid leukaemia. *Nature*, **315**, 726-730.
- Bowen, D.T., Frew, M.E., Hills, R., Gale, R.E., Wheatley, K., Groves, M.J., Langabeer, S.E., Kottaridis, P.D., Moorman, A.V., Burnett, A.K. & Linch, D.C. (2005) RAS mutation in acute myeloid leukemia is associated with distinct cytogenetic subgroups but does not influence outcome in patients < 60 yrs. *Blood*, **106**, 2113-2119.
- Brandts, C.H., Sargin, B., Rode, M., Biermann, C., Lindtner, B., Schwable, J., Buerger, H., Muller-Tidow, C., Choudhary, C., McMahon, M., Berdel, W.E. & Serve, H. (2005) Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. *Cancer Res*, **65**, 9643-9650.

- Brown, P., Meshinchi, S., Levis, M., Alonzo, T.A., Gerbing, R., Lange, B., Arceci, R. & Small, D. (2004) Pediatric AML primary samples with FLT3/ITD mutations are preferentially killed by FLT3 inhibition. *Blood*, **104**, 1841-1849.
- Burnett, A.K. (2002a) Acute myeloid leukemia: treatment of adults under 60 years. *Rev Clin Exp Hematol*, **6**, 26-45; discussion 86-27.
- Burnett, A.K. (2002b) Current controversies: which patients with acute myeloid leukaemia should receive a bone marrow transplantation?--an adult treater's view. *Br J Haematol*, **118**, 357-364.
- Burnett, A.K. & Mohite, U. (2006) Treatment of older patients with acute myeloid leukemia--new agents. *Semin Hematol*, **43**, 96-106.
- Buza-Vidas, N., Cheng, M., Duarte, S., Nozad, H., Jacobsen, S.E. & Sitnicka, E. (2007) Crucial role of FLT3 ligand in immune reconstitution after bone marrow transplantation and high-dose chemotherapy. *Blood*, **110**, 424-432.
- Caligiuri, M.A., Briesewitz, R., Yu, J., Wang, L., Wei, M., Arnoczky, K.J., Marburger, T.B., Wen, J., Perrotti, D., Bloomfield, C.D. & Whitman, S.P. (2007) Novel c-CBL and CBL-b ubiquitin ligase mutations in human acute myeloid leukemia. *Blood*, **110**, 1022-1024.
- Callens, C., Chevret, S., Cayuela, J.M., Cassinat, B., Raffoux, E., de Botton, S., Thomas, X., Guerci, A., Fegueux, N., Pigneux, A., Stoppa, A.M., Lamy, T., Rigal-Huguet, F., Vekhoff, A., Meyer-Monard, S., Ferrand, A., Sanz, M., Chomienne, C., Fenaux, P. & Dombret, H. (2005) Prognostic implication of FLT3 and Ras gene mutations in patients with acute promyelocytic leukemia (APL): a retrospective study from the European APL Group. *Leukemia*, **19**, 1153-1160.
- Capon, D.J., Chen, E.Y., Levinson, A.D., Seeburg, P.H. & Goeddel, D.V. (1983) Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature*, **302**, 33-37.
- Care, R.S., Valk, P.J., Goodeve, A.C., Abu-Duhier, F.M., Geertsma-Kleinekoort, W.M., Wilson, G.A., Gari, M.A., Peake, I.R., Lowenberg, B. & Reilly, J.T. (2003) Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. *Br J Haematol*, **121**, 775-777.
- Cassileth, P.A., Harrington, D.P., Appelbaum, F.R., Lazarus, H.M., Rowe, J.M., Paietta, E., Willman, C., Hurd, D.D., Bennett, J.M., Blume, K.G., Head, D.R. & Wiernik, P.H. (1998) Chemotherapy compared with autologous or allogeneic bone marrow transplantation in the management of acute myeloid leukemia in first remission. *N Engl J Med*, **339**, 1649-1656.
- Casteran, N., Rottapel, R., Beslu, N., Lecocq, E., Birnbaum, D. & Dubreuil, P. (1994) Analysis of the mitogenic pathway of the FLT3 receptor and characterization in its C terminal region of a specific binding site for the PI3' kinase. *Cell Mol Biol (Noisy-le-grand)*, **40**, 443-456.
- Castilla, L.H., Garrett, L., Adya, N., Orlic, D., Dutra, A., Anderson, S., Owens, J., Eckhaus, M., Bodine, D. & Liu, P.P. (1999) The fusion gene Cbfb-MYH11 blocks myeloid differentiation and predisposes mice to acute myelomonocytic leukaemia. *Nat Genet*, **23**, 144-146.
- Chillon, M.C., Fernandez, C., Garcia-Sanz, R., Balanzategui, A., Ramos, F., Fernandez-Calvo, J., Gonzalez, M. & Miguel, J.F. (2004) FLT3-activating mutations are associated with poor prognostic features in AML at diagnosis but they are not an independent prognostic factor. *Hematol J*, **5**, 239-246.
- Chou, T.C. & Talalay, P. (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*, **22**, 27-55.

- Choudhary, C., Muller-Tidow, C., Berdel, W.E. & Serve, H. (2005a) Signal transduction of oncogenic Flt3. *Int J Hematol*, **82**, 93-99.
- Choudhary, C., Schwable, J., Brandts, C., Tickenbrock, L., Sargin, B., Kindler, T., Fischer, T., Berdel, W.E., Muller-Tidow, C. & Serve, H. (2005b) AML-associated Flt3 kinase domain mutations show signal transduction differences in comparison to Flt3 ITD mutations. *Blood*, **106**, 265-273.
- Chung, K.Y., Morrone, G., Schuringa, J.J., Wong, B., Dorn, D.C. & Moore, M.A. (2005) Enforced expression of an Flt3 internal tandem duplication in human CD34+ cells confers properties of self-renewal and enhanced erythropoiesis. *Blood*, **105**, 77-84.
- Clarkson, B., Ohkita, T., Ota, K. & Fried, J. (1967) Studies of cellular proliferation in human leukemia. I. Estimation of growth rates of leukemic and normal hematopoietic cells in two adults with acute leukemia given single injections of tritiated thymidine. *J Clin Invest*, **46**, 506-529.
- Cools, J., Mentens, N., Furet, P., Fabbro, D., Clark, J.J., Griffin, J.D., Marynen, P. & Gilliland, D.G. (2004) Prediction of resistance to small molecule FLT3 inhibitors: implications for molecularly targeted therapy of acute leukemia. *Cancer Res*, **64**, 6385-6389.
- Cornelissen, J.J., van Putten, W.L., Verdonck, L.F., Theobald, M., Jacky, E., Daenen, S.M., van Marwijk Kooy, M., Wijermans, P., Schouten, H., Huijgens, P.C., van der Lelie, H., Fey, M., Ferrant, A., Maertens, J., Gratwohl, A. & Lowenberg, B. (2007) Results of a HOVON/SAKK donor versus no-donor analysis of myeloablative HLA-identical sibling stem cell transplantation in first remission acute myeloid leukemia in young and middle-aged adults: benefits for whom? *Blood*, **109**, 3658-3666.
- Coustan-Smith, E., Sancho, J., Hancock, M.L., Boyett, J.M., Behm, F.G., Raimondi, S.C., Sandlund, J.T., Rivera, G.K., Rubnitz, J.E., Ribeiro, R.C., Pui, C.H. & Campana, D. (2000) Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. *Blood*, **96**, 2691-2696.
- Croce, C.M. (2008) Oncogenes and cancer. *N Engl J Med*, **358**, 502-511.
- Croce, C.M., Thierfelder, W., Erikson, J., Nishikura, K., Finan, J., Lenoir, G.M. & Nowell, P.C. (1983) Transcriptional activation of an unarranged and untranslocated c-myc oncogene by translocation of a C lambda locus in Burkitt. *Proc Natl Acad Sci U S A*, **80**, 6922-6926.
- Dalla-Favera, R., Bregni, M., Erikson, J., Patterson, D., Gallo, R.C. & Croce, C.M. (1982) Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc Natl Acad Sci U S A*, **79**, 7824-7827.
- De Angelo, D.J., Stone, R.M., Heaney, M.L., Nimer, S.D., Paquette, R.L., Klisovic, R.B., Caligiuri, M.A., Cooper, M.R., Lecerf, J.M., Karol, M.D., Sheng, S., Holford, N., Curtin, P.T., Druker, B.J. & Heinrich, M.C. (2006) Phase I clinical results with tandutinib (MLN518), a novel FLT3 antagonist, in patients with acute myelogenous leukemia or high-risk myelodysplastic syndrome: safety, pharmacokinetics, and pharmacodynamics. *Blood*, **108**, 3674-3681.
- Decker, J., Fiedler, W., Samalecos, A. & Hossfeld, D.K. (1995) Absence of point mutations in the extracellular domain of the alpha subunit of the GM-CSF receptor in a series of patients with acute myeloid leukemia (AML). *Leukemia*, **9**, 185-188.
- Dicker, F., Haferlach, C., Kern, W., Haferlach, T. & Schnittger, S. (2007) Trisomy 13 is strongly associated with AML1/RUNX1 mutations and increased FLT3-expression in acute myeloid leukemia. *Blood*, **110**, 1308-1316.

- Dohner, K., Tobis, K., Ulrich, R., Frohling, S., Benner, A., Schlenk, R.F. & Dohner, H. (2002) Prognostic significance of partial tandem duplications of the MLL gene in adult patients 16 to 60 years old with acute myeloid leukemia and normal cytogenetics: a study of the Acute Myeloid Leukemia Study Group Ulm. *J Clin Oncol*, **20**, 3254-3261.
- Dong, J., McPherson, C.M. & Stambrook, P.J. (2002) Flt-3 ligand: a potent dendritic cell stimulator and novel antitumor agent. *Cancer Biol Ther*, **1**, 486-489.
- Dosil, M., Wang, S. & Lemischka, I.R. (1993) Mitogenic signalling and substrate specificity of the Flk2/Flt3 receptor tyrosine kinase in fibroblasts and interleukin 3-dependent hematopoietic cells. *Mol Cell Biol*, **13**, 6572-6585.
- Drexler, H.G. (1996) Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. *Leukemia*, **10**, 588-599.
- Druker, B.J., Talpaz, M., Resta, D.J., Peng, B., Buchdunger, E., Ford, J.M., Lydon, N.B., Kantarjian, H., Capdeville, R., Ohno-Jones, S. & Sawyers, C.L. (2001) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*, **344**, 1031-1037.
- Druker, B.J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G.M., Fanning, S., Zimmermann, J. & Lydon, N.B. (1996) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med*, **2**, 561-566.
- Esteve, J., Escoda, L., Martin, G., Rubio, V., Diaz-Mediavilla, J., Gonzalez, M., Rivas, C., Alvarez, C., Gonzalez San Miguel, J.D., Brunet, S., Tomas, J.F., Tormo, M., Sayas, M.J., Sanchez Godoy, P., Colomer, D., Bolufer, P. & Sanz, M.A. (2007) Outcome of patients with acute promyelocytic leukemia failing to front-line treatment with all-trans retinoic acid and anthracycline-based chemotherapy (PETHEMA protocols LPA96 and LPA99): benefit of an early intervention. *Leukemia*, **21**, 446-452.
- Falini, B., Mecucci, C., Tiacci, E., Alcalay, M., Rosati, R., Pasqualucci, L., La Starza, R., Diverio, D., Colombo, E., Santucci, A., Bigerna, B., Pacini, R., Pucciarini, A., Liso, A., Vignetti, M., Fazi, P., Meani, N., Pettirossi, V., Saglio, G., Mandelli, F., Lo-Coco, F., Pelicci, P.G. & Martelli, M.F. (2005) Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med*, **352**, 254-266.
- Falini, B., Nicoletti, I., Martelli, M.F. & Mecucci, C. (2007) Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+ AML): biologic and clinical features. *Blood*, **109**, 874-885.
- Fenski, R., Flesch, K., Serve, S., Mizuki, M., Oelmann, E., Kratz-Albers, K., Kienast, J., Leo, R., Schwartz, S., Berdel, W.E. & Serve, H. (2000) Constitutive activation of FLT3 in acute myeloid leukaemia and its consequences for growth of 32D cells. *Br J Haematol*, **108**, 322-330.
- Fiedler, W., Mesters, R., Tinnefeld, H., Loges, S., Staib, P., Duhrsen, U., Flasshove, M., Ottmann, O.G., Jung, W., Cavalli, F., Kuse, R., Thomalla, J., Serve, H., O'Farrell, A.M., Jacobs, M., Brega, N.M., Scigalla, P., Hossfeld, D.K. & Berdel, W.E. (2003) A phase 2 clinical study of SU5416 in patients with refractory acute myeloid leukemia. *Blood*, **102**, 2763-2767.
- Fiedler, W., Serve, H., Dohner, H., Schwittay, M., Ottmann, O.G., O'Farrell, A.M., Bello, C.L., Allred, R., Manning, W.C., Cherrington, J.M., Louie, S.G., Hong, W., Brega, N.M., Massimini, G., Scigalla, P., Berdel, W.E. & Hossfeld, D.K. (2005) A phase 1 study of SU11248 in the treatment of patients with refractory or resistant acute myeloid leukemia (AML) or not amenable to conventional therapy for the disease. *Blood*, **105**, 986-993.
- Fitzgibbon, J., Smith, L.L., Raghavan, M., Smith, M.L., Debernardi, S., Skoulakis, S., Lillington, D., Lister, T.A. & Young, B.D. (2005) Association between acquired

- uniparental disomy and homozygous gene mutation in acute myeloid leukemias. *Cancer Res*, **65**, 9152-9154.
- Forbes, L.V., Gale, R.E., Pizzey, A., Pouwels, K., Nathwani, A. & Linch, D.C. (2002) An activating mutation in the transmembrane domain of the granulocyte colony-stimulating factor receptor in patients with acute myeloid leukemia. *Oncogene*, **21**, 5981-5989.
- Freeburn, R.W., Gale, R.E. & Linch, D.C. (1998) Activating point mutations in the betaC chain of the GM-CSF, IL-3 and IL-5 receptors are not a major contributory factor in the pathogenesis of acute myeloid leukaemia. *Br J Haematol*, **103**, 66-71.
- Frohling, S., Schlenk, R.F., Breitnick, J., Benner, A., Kreitmeier, S., Tobis, K., Dohner, H. & Dohner, K. (2002) Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. *Blood*, **100**, 4372-4380.
- Frohling, S., Schlenk, R.F., Stolze, I., Bihlmayr, J., Benner, A., Kreitmeier, S., Tobis, K., Dohner, H. & Dohner, K. (2004) CEBPA mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. *J Clin Oncol*, **22**, 624-633.
- Frohling, S., Scholl, C., Levine, R.L., Loriaux, M., Boggon, T.J., Bernard, O.A., Berger, R., Dohner, H., Dohner, K., Ebert, B.L., Teckie, S., Golub, T.R., Jiang, J., Schittenhelm, M.M., Lee, B.H., Griffin, J.D., Stone, R.M., Heinrich, M.C., Deininger, M.W., Druker, B.J. & Gilliland, D.G. (2007) Identification of driver and passenger mutations of FLT3 by high-throughput DNA sequence analysis and functional assessment of candidate alleles. *Cancer Cell*, **12**, 501-513.
- Furitsu, T., Tsujimura, T., Tono, T., Ikeda, H., Kitayama, H., Koshimizu, U., Sugahara, H., Butterfield, J.H., Ashman, L.K., Kanayama, Y. & et al. (1993) Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. *J Clin Invest*, **92**, 1736-1744.
- Gale, R.E., Green, C., Allen, C., Mead, A.J., Burnett, A.K., Hills, R.K. & Linch, D.C. (2007) The impact of FLT3 internal tandem duplication mutant level, number, size and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. *Blood*, **111**, 2776-2784.
- Gale, R.E., Hills, R., Kottaridis, P.D., Srirangan, S., Wheatley, K., Burnett, A.K. & Linch, D.C. (2005a) No evidence that FLT3 status should be considered as an indicator for transplantation in acute myeloid leukemia (AML): an analysis of 1135 patients, excluding acute promyelocytic leukemia, from the UK MRC AML10 and 12 trials. *Blood*, **106**, 3658-3665.
- Gale, R.E., Hills, R., Pizzey, A.R., Kottaridis, P.D., Swirsky, D., Gilkes, A.F., Nugent, E., Mills, K.I., Wheatley, K., Solomon, E., Burnett, A.K., Linch, D.C. & Grimwade, D. (2005b) The relationship between FLT3 mutation status, biological characteristics and response to targeted therapy in acute promyelocytic leukemia. *Blood*, **106**, 3768-3776.
- Gazit, A., Yee, K., Uecker, A., Bohmer, F.D., Sjoblom, T., Ostman, A., Waltenberger, J., Golomb, G., Banai, S., Heinrich, M.C. & Levitzki, A. (2003) Tricyclic quinoxalines as potent kinase inhibitors of PDGFR kinase, Flt3 and Kit. *Bioorg Med Chem*, **11**, 2007-2018.
- Giles, F.J., Stopeck, A.T., Silverman, L.R., Lancet, J.E., Cooper, M.A., Hannah, A.L., Cherrington, J.M., O'Farrell, A.M., Yuen, H.A., Louie, S.G., Hong, W., Cortes, J.E., Verstovsek, S., Albitar, M., O'Brien, S.M., Kantarjian, H.M. & Karp, J.E. (2003) SU5416, a small molecule tyrosine kinase receptor inhibitor, has biologic

- activity in patients with refractory acute myeloid leukemia or myelodysplastic syndromes. *Blood*, **102**, 795-801.
- Gilliland, D.G. & Griffin, J.D. (2002a) Role of FLT3 in leukemia. *Curr Opin Hematol*, **9**, 274-281.
- Gilliland, D.G. & Griffin, J.D. (2002b) The roles of FLT3 in hematopoiesis and leukemia. *Blood*, **100**, 1532-1542.
- Giralt, S.A., Arora, M., Goldman, J.M., Lee, S.J., Maziarz, R.T., McCarthy, P.L., Sobocinski, K.A. & Horowitz, M.M. (2007) Impact of imatinib therapy on the use of allogeneic haematopoietic progenitor cell transplantation for the treatment of chronic myeloid leukaemia. *Br J Haematol*, **137**, 461-467.
- Grandage, V.L., Everington, T., Linch, D.C. & Khwaja, A. (2006) Go6976 is a potent inhibitor of the JAK 2 and FLT3 tyrosine kinases with significant activity in primary acute myeloid leukaemia cells. *Br J Haematol*, **135**, 303-316.
- Grandage, V.L., Gale, R.E., Linch, D.C. & Khwaja, A. (2005) PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kappaB, Mapkinase and p53 pathways. *Leukemia*, **19**, 586-594.
- Greenberger, J.S., Sakakeeny, M.A., Humphries, R.K., Eaves, C.J. & Eckner, R.J. (1983) Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. *Proc Natl Acad Sci U S A*, **80**, 2931-2935.
- Griessinger, E., Imbert, V., Lagadec, P., Gonthier, N., Dubreuil, P., Romanelli, A., Dreano, M. & Peyron, J.F. (2007) AS602868, a dual inhibitor of IKK2 and FLT3 to target AML cells. *Leukemia*, **21**, 877-885.
- Griffith, J., Black, J., Faerman, C., Swenson, L., Wynn, M., Lu, F., Lippke, J. & Saxena, K. (2004) The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. *Mol Cell*, **13**, 169-178.
- Grimwade, D., Gorman, P., Duprez, E., Howe, K., Langabeer, S., Oliver, F., Walker, H., Culligan, D., Waters, J., Pomfret, M., Goldstone, A., Burnett, A., Freemont, P., Sheer, D. & Solomon, E. (1997) Characterization of cryptic rearrangements and variant translocations in acute promyelocytic leukemia. *Blood*, **90**, 4876-4885.
- Grimwade, D., Walker, H., Oliver, F., Wheatley, K., Harrison, C., Harrison, G., Rees, J., Hann, I., Stevens, R., Burnett, A. & Goldstone, A. (1998) The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood*, **92**, 2322-2333.
- Grisolano, J.L., Wesselschmidt, R.L., Pelicci, P.G. & Ley, T.J. (1997) Altered myeloid development and acute leukemia in transgenic mice expressing PML-RAR alpha under control of cathepsin G regulatory sequences. *Blood*, **89**, 376-387.
- Grundler, R., Miething, C., Thiede, C., Peschel, C. & Duyster, J. (2005) FLT3-ITD and tyrosine kinase domain mutants induce two distinct phenotypes in a murine bone marrow transplant model. *Blood*, **105**, 4792-4799.
- Grundler, R., Thiede, C., Miething, C., Steudel, C., Peschel, C. & Duyster, J. (2003) Sensitivity toward tyrosine kinase inhibitors varies between different activating mutations of the FLT3 receptor. *Blood*, **102**, 646-651.
- Hanawa, H., Kelly, P.F., Nathwani, A.C., Persons, D.A., Vandergriff, J.A., Hargrove, P., Vanin, E.F. & Nienhuis, A.W. (2002) Comparison of various envelope proteins for their ability to pseudotype lentiviral vectors and transduce primitive hematopoietic cells from human blood. *Mol Ther*, **5**, 242-251.
- Hann, I.M., Stevens, R.F., Goldstone, A.H., Rees, J.K., Wheatley, K., Gray, R.G. & Burnett, A.K. (1997) Randomized comparison of DAT versus ADE as induction

- chemotherapy in children and younger adults with acute myeloid leukemia. Results of the Medical Research Council's 10th AML trial (MRC AML10). Adult and Childhood Leukaemia Working Parties of the Medical Research Council. *Blood*, **89**, 2311-2318.
- Harris, N.L., Jaffe, E.S., Diebold, J., Flandrin, G., Muller-Hermelink, H.K., Vardiman, J., Lister, T.A. & Bloomfield, C.D. (1999) World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. *J Clin Oncol*, **17**, 3835-3849.
- Hayakawa, F., Towatari, M., Kiyoi, H., Tanimoto, M., Kitamura, T., Saito, H. & Naoe, T. (2000) Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. *Oncogene*, **19**, 624-631.
- He, L.Z., Tribioli, C., Rivi, R., Peruzzi, D., Pelicci, P.G., Soares, V., Cattoretti, G. & Pandolfi, P.P. (1997) Acute leukemia with promyelocytic features in PML/RARalpha transgenic mice. *Proc Natl Acad Sci U S A*, **94**, 5302-5307.
- Heidel, F., Solem, F.K., Breitenbuecher, F., Lipka, D.B., Kasper, S., Thiede, M.H., Brandts, C., Serve, H., Roesel, J., Giles, F., Feldman, E., Ehninger, G., Schiller, G.J., Nimer, S., Stone, R.M., Wang, Y., Kindler, T., Cohen, P.S., Huber, C. & Fischer, T. (2006) Clinical resistance to the kinase inhibitor PKC412 in acute myeloid leukemia by mutation of Asn-676 in the FLT3 tyrosine kinase domain. *Blood*, **107**, 293-300.
- Higuchi, M., O'Brien, D., Kumaravelu, P., Lenny, N., Yeoh, E.J. & Downing, J.R. (2002) Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. *Cancer Cell*, **1**, 63-74.
- Hong, D., Gupta, R., Ancliff, P., Atzberger, A., Brown, J., Soneji, S., Green, J., Colman, S., Piacibello, W., Buckle, V., Tsuzuki, S., Greaves, M. & Enver, T. (2008) Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia. *Science*, **319**, 336-339.
- Jiang, J., Paez, J.G., Lee, J.C., Bo, R., Stone, R.M., DeAngelo, D.J., Galinsky, I., Wolpin, B.M., Jonasova, A., Herman, P., Fox, E.A., Boggon, T.J., Eck, M.J., Weisberg, E., Griffin, J.D., Gilliland, D.G., Meyerson, M. & Sellers, W.R. (2004) Identifying and characterizing a novel activating mutation of the FLT3 tyrosine kinase in AML. *Blood*, **104**, 1855-1858.
- Jilani, I., Estey, E., Manshuri, T., Caligiuri, M., Keating, M., Giles, F., Thomas, D., Kantarjian, H. & Albitar, M. (2003) Better detection of FLT3 internal tandem duplication using peripheral blood plasma DNA. *Leukemia*, **17**, 114-119.
- Kainz, B., Heintel, D., Marculescu, R., Schwarzing, I., Sperr, W., Le, T., Weltermann, A., Fonatsch, C., Haas, O.A., Mannhalter, C., Lechner, K. & Jaeger, U. (2002) Variable prognostic value of FLT3 internal tandem duplications in patients with de novo AML and a normal karyotype, t(15;17), t(8;21) or inv(16). *Hematol J*, **3**, 283-289.
- Kasper, S., Breitenbuecher, F., Hoehn, Y., Heidel, F., Lipka, D.B., Markova, B., Huber, C., Kindler, T. & Fischer, T. (2008) The kinase inhibitor LS104 induces apoptosis, enhances cytotoxic effects of chemotherapeutic drugs and is targeting the receptor tyrosine kinase FLT3 in acute myeloid leukemia. *Leuk Res*, **32**, 1698-1708.
- Kelly, L.M. & Gilliland, D.G. (2002) Genetics of myeloid leukemias. *Annu Rev Genomics Hum Genet*, **3**, 179-198.

- Kelly, L.M., Kutok, J.L., Williams, I.R., Boulton, C.L., Amaral, S.M., Curley, D.P., Ley, T.J. & Gilliland, D.G. (2002a) PML/RARalpha and FLT3-ITD induce an APL-like disease in a mouse model. *Proc Natl Acad Sci U S A*, **99**, 8283-8288.
- Kelly, L.M., Liu, Q., Kutok, J.L., Williams, I.R., Boulton, C.L. & Gilliland, D.G. (2002b) FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. *Blood*, **99**, 310-318.
- Kelly, L.M., Yu, J.C., Boulton, C.L., Apatira, M., Li, J., Sullivan, C.M., Williams, I., Amaral, S.M., Curley, D.P., Duclos, N., Neuberg, D., Scarborough, R.M., Pandey, A., Hollenbach, S., Abe, K., Lokker, N.A., Gilliland, D.G. & Giese, N.A. (2002c) CT53518, a novel selective FLT3 antagonist for the treatment of acute myelogenous leukemia (AML). *Cancer Cell*, **1**, 421-432.
- Kelly, P.N., Dakic, A., Adams, J.M., Nutt, S.L. & Strasser, A. (2007) Tumor growth need not be driven by rare cancer stem cells. *Science*, **317**, 337.
- Kim, H.G., Kojima, K., Swindle, C.S., Cotta, C.V., Huo, Y., Reddy, V. & Klug, C.A. (2007) FLT3-ITD cooperates with inv(16) to promote progression to acute myeloid leukemia. *Blood*, **111**, 1567-1574.
- Kim, K.T., Baird, K., Ahn, J.Y., Meltzer, P., Lilly, M., Levis, M. & Small, D. (2005) Pim-1 is up-regulated by constitutively activated FLT3 and plays a role in FLT3-mediated cell survival. *Blood*, **105**, 1759-1767.
- Kim, K.T., Levis, M. & Small, D. (2006) Constitutively activated FLT3 phosphorylates BAD partially through pim-1. *Br J Haematol*, **134**, 500-509.
- Kindler, T., Breitenbuecher, F., Kasper, S., Estey, E., Giles, F., Feldman, E., Ehninger, G., Schiller, G., Klimek, V., Nimer, S.D., Gratwohl, A., Choudhary, C.R., Mueller-Tidow, C., Serve, H., Gschaidmeier, H., Cohen, P.S., Huber, C. & Fischer, T. (2005) Identification of a novel activating mutation (Y842C) within the activation loop of FLT3 in patients with acute myeloid leukemia (AML). *Blood*, **105**, 335-340.
- Kirstetter, P., Schuster, M.B., Bereshchenko, O., Moore, S., Dvinge, H., Kurz, E., Theilgaard-Monch, K., Mansson, R., Pedersen, T.A., Pabst, T., Schrock, E., Porse, B.T., Jacobsen, S.E., Bertone, P., Tenen, D.G. & Nerlov, C. (2008) Modeling of C/EBPalpha mutant acute myeloid leukemia reveals a common expression signature of committed myeloid leukemia-initiating cells. *Cancer Cell*, **13**, 299-310.
- Kitayama, H., Kanakura, Y., Furitsu, T., Tsujimura, T., Oritani, K., Ikeda, H., Sugahara, H., Mitsui, H., Kanayama, Y., Kitamura, Y. & et al. (1995) Constitutively activating mutations of c-kit receptor tyrosine kinase confer factor-independent growth and tumorigenicity of factor-dependent hematopoietic cell lines. *Blood*, **85**, 790-798.
- Kiyoi, H., Naoe, T., Nakano, Y., Yokota, S., Minami, S., Miyawaki, S., Asou, N., Kuriyama, K., Jinnai, I., Shimazaki, C., Akiyama, H., Saito, K., Oh, H., Motoji, T., Omoto, E., Saito, H., Ohno, R. & Ueda, R. (1999) Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood*, **93**, 3074-3080.
- Kiyoi, H., Naoe, T., Yokota, S., Nakao, M., Minami, S., Kuriyama, K., Takeshita, A., Saito, K., Hasegawa, S., Shimodaira, S., Tamura, J., Shimazaki, C., Matsue, K., Kobayashi, H., Arima, N., Suzuki, R., Morishita, H., Saito, H., Ueda, R. & Ohno, R. (1997) Internal tandem duplication of FLT3 associated with leukocytosis in acute promyelocytic leukemia. Leukemia Study Group of the Ministry of Health and Welfare (Kohseisho). *Leukemia*, **11**, 1447-1452.

- Kiyoi, H., Ohno, R., Ueda, R., Saito, H. & Naoe, T. (2002) Mechanism of constitutive activation of FLT3 with internal tandem duplication in the juxtamembrane domain. *Oncogene*, **21**, 2555-2563.
- Kiyoi, H., Shiotsu, Y., Ozeki, K., Yamaji, S., Kosugi, H., Umehara, H., Shimizu, M., Arai, H., Ishii, K., Akinaga, S. & Naoe, T. (2007) A novel FLT3 inhibitor FI-700 selectively suppresses the growth of leukemia cells with FLT3 mutations. *Clin Cancer Res*, **13**, 4575-4582.
- Kiyoi, H., Towatari, M., Yokota, S., Hamaguchi, M., Ohno, R., Saito, H. & Naoe, T. (1998) Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. *Leukemia*, **12**, 1333-1337.
- Knapper, S. (2007) FLT3 inhibition in acute myeloid leukaemia. *Br J Haematol*, **138**, 687-699.
- Knapper, S., Burnett, A.K., Littlewood, T., Kell, W.J., Agrawal, S., Chopra, R., Clark, R., Levis, M.J. & Small, D. (2006a) A phase 2 trial of the FLT3 inhibitor lestaurtinib (CEP701) as first-line treatment for older patients with acute myeloid leukemia not considered fit for intensive chemotherapy. *Blood*, **108**, 3262-3270.
- Knapper, S., Mills, K.I., Gilkes, A.F., Austin, S.J., Walsh, V. & Burnett, A.K. (2006b) The effects of lestaurtinib (CEP701) and PKC412 on primary AML blasts: the induction of cytotoxicity varies with dependence on FLT3 signaling in both FLT3-mutated and wild-type cases. *Blood*, **108**, 3494-3503.
- Koch, S., Jacobi, A., Ryser, M., Ehninger, G. & Thiede, C. (2008) Abnormal Localization and Accumulation of FLT3-ITD, a Mutant Receptor Tyrosine Kinase Involved in Leukemogenesis. *Cells Tissues Organs*, **188**, 225-235.
- Komeno, Y., Kurokawa, M., Imai, Y., Takeshita, M., Matsumura, T., Kubo, K., Yoshino, T., Nishiyama, U., Kuwaki, T., Kubo, K., Osawa, T., Ogawa, S., Chiba, S., Miwa, A. & Hirai, H. (2005) Identification of Ki23819, a highly potent inhibitor of kinase activity of mutant FLT3 receptor tyrosine kinase. *Leukemia*, **19**, 930-935.
- Kottaridis, P.D., Gale, R.E., Frew, M.E., Harrison, G., Langabeer, S.E., Belton, A.A., Walker, H., Wheatley, K., Bowen, D.T., Burnett, A.K., Goldstone, A.H. & Linch, D.C. (2001) The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood*, **98**, 1752-1759.
- Kottaridis, P.D., Gale, R.E., Langabeer, S.E., Frew, M.E., Bowen, D.T. & Linch, D.C. (2002) Studies of FLT3 mutations in paired presentation and relapse samples from patients with acute myeloid leukemia: implications for the role of FLT3 mutations in leukemogenesis, minimal residual disease detection, and possible therapy with FLT3 inhibitors. *Blood*, **100**, 2393-2398.
- Kottaridis, P.D., Gale, R.E. & Linch, D.C. (2003) Flt3 mutations and leukaemia. *Br J Haematol*, **122**, 523-538.
- Krause, D.S. & Van Etten, R.A. (2005) Tyrosine kinases as targets for cancer therapy. *N Engl J Med*, **353**, 172-187.
- Krivtsov, A.V., Twomey, D., Feng, Z., Stubbs, M.C., Wang, Y., Faber, J., Levine, J.E., Wang, J., Hahn, W.C., Gilliland, D.G., Golub, T.R. & Armstrong, S.A. (2006) Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature*, **442**, 818-822.
- Kuchenbauer, F., Schoch, C., Kern, W., Hiddemann, W., Haferlach, T. & Schnittger, S. (2005) Impact of FLT3 mutations and promyelocytic leukaemia-breakpoint on

- clinical characteristics and prognosis in acute promyelocytic leukaemia. *Br J Haematol*, **130**, 196-202.
- Langabeer, S.E., Walker, H., Gale, R.E., Wheatley, K., Burnett, A.K., Goldstone, A.H. & Linch, D.C. (1997a) Frequency of CBF beta/MYH11 fusion transcripts in patients entered into the U.K. MRC AML trials. The MRC Adult Leukaemia Working Party. *Br J Haematol*, **96**, 736-739.
- Langabeer, S.E., Walker, H., Rogers, J.R., Burnett, A.K., Wheatley, K., Swirsky, D., Goldstone, A.H. & Linch, D.C. (1997b) Incidence of AML1/ETO fusion transcripts in patients entered into the MRC AML trials. MRC Adult Leukaemia Working Party. *Br J Haematol*, **99**, 925-928.
- Lanotte, M., Martin-Thouvenin, V., Najman, S., Balerini, P., Valensi, F. & Berger, R. (1991) NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood*, **77**, 1080-1086.
- Lavagna-Sevenier, C., Marchetto, S., Birnbaum, D. & Rosnet, O. (1998a) The CBL-related protein CBLB participates in FLT3 and interleukin-7 receptor signal transduction in pro-B cells. *J Biol Chem*, **273**, 14962-14967.
- Lavagna-Sevenier, C., Marchetto, S., Birnbaum, D. & Rosnet, O. (1998b) FLT3 signaling in hematopoietic cells involves CBL, SHC and an unknown P115 as prominent tyrosine-phosphorylated substrates. *Leukemia*, **12**, 301-310.
- Le Couedic, J.P., Mitjavila, M.T., Villeval, J.L., Feger, F., Gobert, S., Mayeux, P., Casadevall, N. & Vainchenker, W. (1996) Missense mutation of the erythropoietin receptor is a rare event in human erythroid malignancies. *Blood*, **87**, 1502-1511.
- Lee, B.H., Tothova, Z., Levine, R.L., Anderson, K., Buza-Vidas, N., Cullen, D.E., McDowell, E.P., Adelsperger, J., Frohling, S., Huntly, B.J., Beran, M., Jacobsen, S.E. & Gilliland, D.G. (2007) FLT3 Mutations Confer Enhanced Proliferation and Survival Properties to Multipotent Progenitors in a Murine Model of Chronic Myelomonocytic Leukemia. *Cancer Cell*, **12**, 367-380.
- Lee, B.H., Williams, I.R., Anastasiadou, E., Boulton, C.L., Joseph, S.W., Amaral, S.M., Curley, D.P., Duclos, N., Huntly, B.J., Fabbro, D., Griffin, J.D. & Gilliland, D.G. (2005) FLT3 internal tandem duplication mutations induce myeloproliferative or lymphoid disease in a transgenic mouse model. *Oncogene*, **24**, 7882-7892.
- Levis, M., Allebach, J., Tse, K.F., Zheng, R., Baldwin, B.R., Smith, B.D., Jones-Bolin, S., Ruggeri, B., Dionne, C. & Small, D. (2002) A FLT3-targeted tyrosine kinase inhibitor is cytotoxic to leukemia cells in vitro and in vivo. *Blood*, **99**, 3885-3891.
- Levis, M., Brown, P., Smith, B.D., Stine, A., Pham, R., Stone, R., Deangelo, D., Galinsky, I., Giles, F., Estey, E., Kantarjian, H., Cohen, P., Wang, Y., Roesel, J., Karp, J.E. & Small, D. (2006) Plasma inhibitory activity (PIA): a pharmacodynamic assay reveals insights into the basis for cytotoxic response to FLT3 inhibitors. *Blood*, **108**, 3477-3483.
- Levis, M., Murphy, K.M., Pham, R., Kim, K.T., Stine, A., Li, L., McNiece, I., Smith, B.D. & Small, D. (2005a) Internal tandem duplications of the FLT3 gene are present in leukemia stem cells. *Blood*, **106**, 673-680.
- Levis, M., Pham, R., Smith, B.D. & Small, D. (2004) In vitro studies of a FLT3 inhibitor combined with chemotherapy: sequence of administration is important to achieve synergistic cytotoxic effects. *Blood*, **104**, 1145-1150.
- Levis, M. & Small, D. (2003) FLT3: ITDoes matter in leukemia. *Leukemia*, **17**, 1738-1752.
- Levis, M., Smith, B., Beran, M., Baer, M., Erba, H., Cripe, L., Coutre, S., Advani, A., Perl, A., Devetten, M., Stuart, R., Tallman, M., Brown, P., Tremmel, L. & D., S.

- (2005b) A Randomized, Open-Label Study of Lestaurtinib (CEP-701), an Oral FLT3 Inhibitor, Administered in Sequence with Chemotherapy in Patients with Relapsed AML Harboring FLT3 Activating Mutations: Clinical Response Correlates with Successful FLT3 Inhibition. *Blood*, **106**, Abstract 403.
- Levis, M., Tse, K.F., Smith, B.D., Garrett, E. & Small, D. (2001) A FLT3 tyrosine kinase inhibitor is selectively cytotoxic to acute myeloid leukemia blasts harboring FLT3 internal tandem duplication mutations. *Blood*, **98**, 885-887.
- Ley, T.J., Mardis, E.R., Ding, L., Fulton, B., McLellan, M.D., Chen, K., Dooling, D., Dunford-Shore, B.H., McGrath, S., Hickenbotham, M., Cook, L., Abbott, R., Larson, D.E., Koboldt, D.C., Pohl, C., Smith, S., Hawkins, A., Abbott, S., Locke, D., Hillier, L.W., Miner, T., Fulton, L., Magrini, V., Wylie, T., Glasscock, J., Conyers, J., Sander, N., Shi, X., Osborne, J.R., Minx, P., Gordon, D., Chinwalla, A., Zhao, Y., Ries, R.E., Payton, J.E., Westervelt, P., Tomasson, M.H., Watson, M., Baty, J., Ivanovich, J., Heath, S., Shannon, W.D., Nagarajan, R., Walter, M.J., Link, D.C., Graubert, T.A., DiPersio, J.F. & Wilson, R.K. (2008) DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature*, **456**, 66-72.
- Li, L., Piloto, O., Kim, K.T., Ye, Z., Nguyen, H.B., Yu, X., Levis, M., Cheng, L. & Small, D. (2007) FLT3/ITD expression increases expansion, survival and entry into cell cycle of human haematopoietic stem/progenitor cells. *Br J Haematol*, **137**, 64-75.
- Libura, M., Asnafi, V., Tu, A., Delabesse, E., Tigaud, I., Cymbalista, F., Bennaceur-Griscelli, A., Villarese, P., Solbu, G., Hagemeijer, A., Beldjord, K., Hermine, O. & Macintyre, E. (2003) FLT3 and MLL intragenic abnormalities in AML reflect a common category of genotoxic stress. *Blood*, **102**, 2198-2204.
- Litzow, M.R. (2005) More flitting about FLT3. *Blood*, **106**, 3331-3332.
- Liu, W., Smith, D.I., Rechtzigel, K.J., Thibodeau, S.N. & James, C.D. (1998) Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. *Nucleic Acids Res*, **26**, 1396-1400.
- Lo-Coco, F. & Ammatuna, E. (2006) The biology of acute promyelocytic leukemia and its impact on diagnosis and treatment. *Hematology Am Soc Hematol Educ Program*, 156-161, 514.
- Lo-Coco, F., Breccia, M. & Diverio, D. (2003) The importance of molecular monitoring in acute promyelocytic leukaemia. *Best Pract Res Clin Haematol*, **16**, 503-520.
- Longley, B.J., Jr., Metcalfe, D.D., Tharp, M., Wang, X., Tyrrell, L., Lu, S.Z., Heitjan, D. & Ma, Y. (1999) Activating and dominant inactivating c-KIT catalytic domain mutations in distinct clinical forms of human mastocytosis. *Proc Natl Acad Sci U S A*, **96**, 1609-1614.
- Longmore, G.D. & Lodish, H.F. (1991) An activating mutation in the murine erythropoietin receptor induces erythroleukemia in mice: a cytokine receptor superfamily oncogene. *Cell*, **67**, 1089-1102.
- Lu, Y., Kitaura, J., Oki, T., Komeno, Y., Ozaki, K., Kiyono, M., Kumagai, H., Nakajima, H., Nosaka, T., Aburatani, H. & Kitamura, T. (2007) Identification of TSC-22 as a potential tumor suppressor that is upregulated by Flt3-D835V but not Flt3-ITD. *Leukemia*, **21**, 2246-2257.
- Luc, S., Buza-Vidas, N. & Jacobsen, S.E. (2007) Biological and molecular evidence for existence of lymphoid-primed multipotent progenitors. *Ann N Y Acad Sci*, **1106**, 89-94.
- Lyman, S.D., James, L., Vanden Bos, T., de Vries, P., Brasel, K., Gliniak, B., Hollingsworth, L.T., Picha, K.S., McKenna, H.J., Splett, R.R. & et al. (1993) Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. *Cell*, **75**, 1157-1167.

- Mackarechtschian, K., Hardin, J.D., Moore, K.A., Boast, S., Goff, S.P. & Lemischka, I.R. (1995) Targeted disruption of the *flk2/flt3* gene leads to deficiencies in primitive hematopoietic progenitors. *Immunity*, **3**, 147-161.
- MacKenzie, K.L., Dolnikov, A., Millington, M., Shounan, Y. & Symonds, G. (1999) Mutant N-ras induces myeloproliferative disorders and apoptosis in bone marrow repopulated mice. *Blood*, **93**, 2043-2056.
- Marchetto, S., Fournier, E., Beslu, N., Aurran-Schleinitz, T., Dubreuil, P., Borg, J.P., Birnbaum, D. & Rosnet, O. (1999) SHC and SHIP phosphorylation and interaction in response to activation of the FLT3 receptor. *Leukemia*, **13**, 1374-1382.
- Marcucci, G., Baldus, C.D., Ruppert, A.S., Radmacher, M.D., Mrozek, K., Whitman, S.P., Kolitz, J.E., Edwards, C.G., Vardiman, J.W., Powell, B.L., Baer, M.R., Moore, J.O., Perrotti, D., Caligiuri, M.A., Carroll, A.J., Larson, R.A., de la Chapelle, A. & Bloomfield, C.D. (2005) Overexpression of the ETS-related gene, *ERG*, predicts a worse outcome in acute myeloid leukemia with normal karyotype: a Cancer and Leukemia Group B study. *J Clin Oncol*, **23**, 9234-9242.
- Marcucci, G., Maharry, K., Whitman, S.P., Vukosavljevic, T., Paschka, P., Langer, C., Mrozek, K., Baldus, C.D., Carroll, A.J., Powell, B.L., Kolitz, J.E., Larson, R.A. & Bloomfield, C.D. (2007) High expression levels of the ETS-related gene, *ERG*, predict adverse outcome and improve molecular risk-based classification of cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B Study. *J Clin Oncol*, **25**, 3337-3343.
- Martin, G.S. (1970) Rous sarcoma virus: a function required for the maintenance of the transformed state. *Nature*, **227**, 1021-1023.
- Marx, J.L. (1989) Cancer gene research wins medicine Nobel. *Science*, **246**, 326-327.
- Mathews, V., Thomas, M., Srivastava, V.M., George, B., Srivastava, A. & Chandy, M. (2007) Impact of FLT3 mutations and secondary cytogenetic changes on the outcome of patients with newly diagnosed acute promyelocytic leukemia treated with a single agent arsenic trioxide regimen. *Haematologica*, **92**, 994-995.
- Matthews, W., Jordan, C.T., Wiegand, G.W., Pardoll, D. & Lemischka, I.R. (1991) A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. *Cell*, **65**, 1143-1152.
- McCoy, M.S., Toole, J.J., Cunningham, J.M., Chang, E.H., Lowy, D.R. & Weinberg, R.A. (1983) Characterization of a human colon/lung carcinoma oncogene. *Nature*, **302**, 79-81.
- Mead, A.J., Linch, D.C., Hills, R.K., Wheatley, K., Burnett, A.K. & Gale, R.E. (2007) FLT3 tyrosine kinase domain mutations are biologically distinct from and have a significantly more favorable prognosis than FLT3 internal tandem duplications in patients with acute myeloid leukemia. *Blood*, **110**, 1262-1270.
- Meshinchi, S., Alonzo, T.A., Stirewalt, D.L., Zwaan, M., Zimmerman, M., Reinhardt, D., Kaspers, G.J., Heerema, N.A., Gerbing, R., Lange, B.J. & Radich, J.P. (2006) Clinical implications of FLT3 mutations in pediatric AML. *Blood*, **108**, 3654-3661.
- Milligan, D.W., Grimwade, D., Cullis, J.O., Bond, L., Swirsky, D., Craddock, C., Kell, J., Homewood, J., Campbell, K., McGinley, S., Wheatley, K. & Jackson, G. (2006) Guidelines on the management of acute myeloid leukaemia in adults. *Br J Haematol*, **135**, 450-474.
- Mills, K.I., Gilkes, A.F., Walsh, V., Sweeney, M. & Gale, R. (2005) Rapid and sensitive detection of internal tandem duplication and activating loop mutations of FLT3. *Br J Haematol*, **130**, 203-208.

- Mills, K.I., Walsh, V., Gilkes, A.F., Agrawal, S.G. & Knapper, S. (2006) Novel observation of three FLT3 codons mutated in tandem in an elderly acute myeloid leukaemia patient. *Br J Haematol*, **132**, 116-117.
- Minami, Y., Kiyoi, H., Yamamoto, Y., Yamamoto, K., Ueda, R., Saito, H. & Naoe, T. (2002) Selective apoptosis of tandemly duplicated FLT3-transformed leukemia cells by Hsp90 inhibitors. *Leukemia*, **16**, 1535-1540.
- Minami, Y., Yamamoto, K., Kiyoi, H., Ueda, R., Saito, H. & Naoe, T. (2003) Different antiapoptotic pathways between wild-type and mutated FLT3: insights into therapeutic targets in leukemia. *Blood*, **102**, 2969-2975.
- Mistry, A.R., Pedersen, E.W., Solomon, E. & Grimwade, D. (2003) The molecular pathogenesis of acute promyelocytic leukaemia: implications for the clinical management of the disease. *Blood Rev*, **17**, 71-97.
- Mizuki, M., Fenski, R., Halfter, H., Matsumura, I., Schmidt, R., Muller, C., Gruning, W., Kratz-Albers, K., Serve, S., Steur, C., Buchner, T., Kienast, J., Kanakura, Y., Berdel, W.E. & Serve, H. (2000) Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. *Blood*, **96**, 3907-3914.
- Mizuki, M., Schwable, J., Steur, C., Choudhary, C., Agrawal, S., Sargin, B., Steffen, B., Matsumura, I., Kanakura, Y., Bohmer, F.D., Muller-Tidow, C., Berdel, W.E. & Serve, H. (2003) Suppression of myeloid transcription factors and induction of STAT response genes by AML-specific Flt3 mutations. *Blood*, **101**, 3164-3173.
- Moore, M.A., Dorn, D.C., Schuringa, J.J., Chung, K.Y. & Morrone, G. (2007) Constitutive activation of Flt3 and STAT5A enhances self-renewal and alters differentiation of hematopoietic stem cells. *Exp Hematol*, **35**, 105-116.
- Moore, T.A. & Zlotnik, A. (1997) Differential effects of Flk-2/Flt-3 ligand and stem cell factor on murine thymic progenitor cells. *J Immunol*, **158**, 4187-4192.
- Moreno, I., Martin, G., Bolufer, P., Barragan, E., Rueda, E., Roman, J., Fernandez, P., Leon, P., Mena, A., Cervera, J., Torres, A. & Sanz, M.A. (2003) Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia. *Haematologica*, **88**, 19-24.
- Mrozek, K., Marcucci, G., Paschka, P., Whitman, S.P. & Bloomfield, C.D. (2007) Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification? *Blood*, **109**, 431-448.
- Murata, K., Kumagai, H., Kawashima, T., Tamitsu, K., Irie, M., Nakajima, H., Suzu, S., Shibuya, M., Kamihira, S., Nosaka, T., Asano, S. & Kitamura, T. (2003) Selective cytotoxic mechanism of GTP-14564, a novel tyrosine kinase inhibitor in leukemia cells expressing a constitutively active Fms-like tyrosine kinase 3 (FLT3). *J Biol Chem*, **278**, 32892-32898.
- Nakano, Y., Kiyoi, H., Miyawaki, S., Asou, N., Ohno, R., Saito, H. & Naoe, T. (1999) Molecular evolution of acute myeloid leukaemia in relapse: unstable N-ras and FLT3 genes compared with p53 gene. *Br J Haematol*, **104**, 659-664.
- Nakao, M., Yokota, S., Iwai, T., Kaneko, H., Horiike, S., Kashima, K., Sonoda, Y., Fujimoto, T. & Misawa, S. (1996) Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia*, **10**, 1911-1918.
- Neben, K., Schnittger, S., Brors, B., Tews, B., Kokocinski, F., Haferlach, T., Muller, J., Hahn, M., Hiddemann, W., Eils, R., Lichter, P. & Schoch, C. (2005) Distinct gene expression patterns associated with FLT3- and NRAS-activating mutations in acute myeloid leukemia with normal karyotype. *Oncogene*, **24**, 1580-1588.
- Nishiyama, U., Yoshino, T., Ozai, M., Yoshioka, R., Fujisawa, M., Ogasawara, Y., Kitahori, M., Yoshioka, E., Kubo, K., Komeno, Y., Kurokawa, M., Ogawa, S., Chiba, S., Osawa, T., Kuwaki, T., Hirai, H. & Miwa, A. (2006) Antineoplastic

- effect of a single oral dose of the novel Flt3 inhibitor KRN383 on xenografted human leukemic cells harboring Flt3-activating mutations. *Leuk Res*, **30**, 1541-1546.
- Noguera, N.I., Breccia, M., Divona, M., Diverio, D., Costa, V., De Santis, S., Avvisati, G., Pinazzi, M.B., Petti, M.C., Mandelli, F. & Lo Coco, F. (2002) Alterations of the FLT3 gene in acute promyelocytic leukemia: association with diagnostic characteristics and analysis of clinical outcome in patients treated with the Italian AIDA protocol. *Leukemia*, **16**, 2185-2189.
- O'Brien, S.G., Guilhot, F., Larson, R.A., Gathmann, I., Baccarani, M., Cervantes, F., Cornelissen, J.J., Fischer, T., Hochhaus, A., Hughes, T., Lechner, K., Nielsen, J.L., Rousselot, P., Reiffers, J., Saglio, G., Shepherd, J., Simonsson, B., Gratwohl, A., Goldman, J.M., Kantarjian, H., Taylor, K., Verhoef, G., Bolton, A.E., Capdeville, R. & Druker, B.J. (2003) Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med*, **348**, 994-1004.
- O'Farrell, A.M., Abrams, T.J., Yuen, H.A., Ngai, T.J., Louie, S.G., Yee, K.W., Wong, L.M., Hong, W., Lee, L.B., Town, A., Smolich, B.D., Manning, W.C., Murray, L.J., Heinrich, M.C. & Cherrington, J.M. (2003) SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo. *Blood*, **101**, 3597-3605.
- Pagano, L., Fianchi, L., Caira, M., Rutella, S. & Leone, G. (2007) The role of Gemtuzumab Ozogamicin in the treatment of acute myeloid leukemia patients. *Oncogene*, **26**, 3679-3690.
- Palacios, R. & Steinmetz, M. (1985) Il-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. *Cell*, **41**, 727-734.
- Paschka, P., Marcucci, G., Ruppert, A.S., Whitman, S.P., Mrozek, K., Maharry, K., Langer, C., Baldus, C.D., Zhao, W., Powell, B.L., Baer, M.R., Carroll, A.J., Caligiuri, M.A., Kolitz, J.E., Larson, R.A. & Bloomfield, C.D. (2008) Wilms Tumor 1 Gene Mutations Independently Predict Poor Outcome in Adults With Cytogenetically Normal Acute Myeloid Leukemia: A Cancer and Leukemia Group B Study. *J Clin Oncol*.
- Piloto, O., Levis, M., Huso, D., Li, Y., Li, H., Wang, M.N., Bassi, R., Balderes, P., Ludwig, D.L., Witte, L., Zhu, Z., Hicklin, D.J. & Small, D. (2005) Inhibitory anti-FLT3 antibodies are capable of mediating antibody-dependent cell-mediated cytotoxicity and reducing engraftment of acute myelogenous leukemia blasts in nonobese diabetic/severe combined immunodeficient mice. *Cancer Res*, **65**, 1514-1522.
- Piloto, O., Wright, M., Brown, P., Kim, K.T., Levis, M. & Small, D. (2006) Prolonged exposure to FLT3 inhibitors leads to resistance via activation of parallel signaling pathways. *Blood*, **109**, 1643-1652.
- Pollard, J.A., Alonzo, T.A., Gerbing, R.B., Woods, W.G., Lange, B.J., Sweetser, D.A., Radich, J.P., Bernstein, I.D. & Meshinchi, S. (2006) FLT3 internal tandem duplication in CD34+/CD33- precursors predicts poor outcome in acute myeloid leukemia. *Blood*, **108**, 2764-2769.
- Pratz, K.W., Cortes, J., Roboz, G.J., Rao, N., Arowojolu, O., Stine, A., Shiotsu, Y., Shudo, A., Akinaga, S., Small, D., Karp, J.E. & Levis, M. (2009) A pharmacodynamic study of the FLT3 inhibitor KW-2449 yields insight into the basis for clinical response. *Blood*, Epub ahead of print.
- Preudhomme, C., Sagot, C., Boissel, N., Cayuela, J.M., Tigaud, I., de Botton, S., Thomas, X., Raffoux, E., Lamandin, C., Castaigne, S., Fenaux, P. & Dombret, H. (2002) Favorable prognostic significance of CEBPA mutations in patients

- with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). *Blood*, **100**, 2717-2723.
- Quentmeier, H., Reinhardt, J., Zaborski, M. & Drexler, H.G. (2003) FLT3 mutations in acute myeloid leukemia cell lines. *Leukemia*, **17**, 120-124.
- Radomska, H.S., Basseres, D.S., Zheng, R., Zhang, P., Dayaram, T., Yamamoto, Y., Sternberg, D.W., Lokker, N., Giese, N.A., Bohlander, S.K., Schnittger, S., Delmotte, M.H., Davis, R.J., Small, D., Hiddemann, W., Gilliland, D.G. & Tenen, D.G. (2006) Block of C/EBP alpha function by phosphorylation in acute myeloid leukemia with FLT3 activating mutations. *J Exp Med*, **203**, 371-381.
- Raghavan, M., Lillington, D.M., Skoulakis, S., Debernardi, S., Chaplin, T., Foot, N.J., Lister, T.A. & Young, B.D. (2005) Genome-wide single nucleotide polymorphism analysis reveals frequent partial uniparental disomy due to somatic recombination in acute myeloid leukemias. *Cancer Res*, **65**, 375-378.
- Rangarajan, A., Hong, S.J., Gifford, A. & Weinberg, R.A. (2004) Species- and cell type-specific requirements for cellular transformation. *Cancer Cell*, **6**, 171-183.
- Ratajczak, J., Marlicz, W. & Ratajczak, M.Z. (1995) [The effect of STK-1 receptor (FLK2/FLT3) ligand on human erythropoiesis in vitro. Clinical implications]. *Pol Arch Med Wewn*, **94**, 418-424.
- Ratajczak, M.Z., Ratajczak, J., Ford, J., Kregenow, R., Marlicz, W. & Gewirtz, A.M. (1996) FLT3/FLK-2 (STK-1) Ligand does not stimulate human megakaryopoiesis in vitro. *Stem Cells*, **14**, 146-150.
- Reindl, C., Bagrintseva, K., Vempati, S., Schnittger, S., Ellwart, J.W., Wenig, K., Hopfner, K.P., Hiddemann, W. & Spiekermann, K. (2006) Point mutations in the juxtamembrane domain of FLT3 define a new class of activating mutations in AML. *Blood*, **107**, 3700-3707.
- Rombouts, W.J., Blokland, I., Lowenberg, B. & Ploemacher, R.E. (2000) Biological characteristics and prognosis of adult acute myeloid leukemia with internal tandem duplications in the Flt3 gene. *Leukemia*, **14**, 675-683.
- Rosnet, O., Buhring, H.J., Marchetto, S., Rappold, I., Lavagna, C., Sainty, D., Arnoulet, C., Chabannon, C., Kanz, L., Hannum, C. & Birnbaum, D. (1996) Human FLT3/FLK2 receptor tyrosine kinase is expressed at the surface of normal and malignant hematopoietic cells. *Leukemia*, **10**, 238-248.
- Rosnet, O., Mattei, M.G., Marchetto, S. & Birnbaum, D. (1991) Isolation and chromosomal localization of a novel FMS-like tyrosine kinase gene. *Genomics*, **9**, 380-385.
- Rusten, L.S., Lyman, S.D., Veiby, O.P. & Jacobsen, S.E. (1996) The FLT3 ligand is a direct and potent stimulator of the growth of primitive and committed human CD34+ bone marrow progenitor cells in vitro. *Blood*, **87**, 1317-1325.
- Sanz, M.A. (2006) Treatment of acute promyelocytic leukemia. *Hematology Am Soc Hematol Educ Program*, 147-155.
- Sanz, M.A., Martin, G., Gonzalez, M., Leon, A., Rayon, C., Rivas, C., Colomer, D., Amutio, E., Capote, F.J., Milone, G.A., De La Serna, J., Roman, J., Barragan, E., Bergua, J., Escoda, L., Parody, R., Negri, S., Calasanz, M.J. & Bolufer, P. (2004) Risk-adapted treatment of acute promyelocytic leukemia with all-trans-retinoic acid and anthracycline monochemotherapy: a multicenter study by the PETHEMA group. *Blood*, **103**, 1237-1243.
- Sargin, B., Choudhary, C., Crosetto, N., Schmidt, M.H., Grundler, R., Rensinghoff, M., Thiessen, C., Tickenbrock, L., Schwable, J., Brandts, C., August, B., Koschmieder, S., Bandi, S.R., Duyster, J., Berdel, W.E., Muller-Tidow, C., Dikic, I. & Serve, H. (2007) Flt3-dependent transformation by inactivating c-Cbl mutations in AML. *Blood*, **110**, 1004-1012.

- Scheijen, B., Ngo, H.T., Kang, H. & Griffin, J.D. (2004) FLT3 receptors with internal tandem duplications promote cell viability and proliferation by signaling through Foxo proteins. *Oncogene*, **23**, 3338-3349.
- Schessler, C., Rawat, V.P., Cusan, M., Deshpande, A., Kohl, T.M., Rosten, P.M., Spiekermann, K., Humphries, R.K., Schnittger, S., Kern, W., Hiddemann, W., Quintanilla-Martinez, L., Bohlander, S.K., Feuring-Buske, M. & Buske, C. (2005) The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice. *J Clin Invest*, **115**, 2159-2168.
- Schittenhelm, M.M., Yee, K.W., Tyner, J.W., McGreevey, L., Haley, A.D., Town, A., Griffith, D.J., Bainbridge, T., Brazier, R.M., O'Farrell, A.M., Cherrington, J.M. & Heinrich, M.C. (2006) FLT3 K663Q is a novel AML-associated oncogenic kinase: Determination of biochemical properties and sensitivity to Sunitinib (SU11248). *Leukemia*, **20**, 2008-2014.
- Schlenk, R.F., Dohner, K., Krauter, J., Frohling, S., Corbacioglu, A., Bullinger, L., Habdank, M., Spath, D., Morgan, M., Benner, A., Schlegelberger, B., Heil, G., Ganser, A. & Dohner, H. (2008) Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med*, **358**, 1909-1918.
- Schnittger, S., Kinkel, U., Schoch, C., Heinecke, A., Haase, D., Haferlach, T., Buchner, T., Wormann, B., Hiddemann, W. & Griesinger, F. (2000) Screening for MLL tandem duplication in 387 unselected patients with AML identify a prognostically unfavorable subset of AML. *Leukemia*, **14**, 796-804.
- Schnittger, S., Schoch, C., Dugas, M., Kern, W., Staib, P., Wuchter, C., Löffler, H., Sauerland, C.M., Serve, H., Buchner, T., Haferlach, T. & Hiddemann, W. (2002) Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood*, **100**, 59-66.
- Schnittger, S., Schoch, C., Kern, W., Hiddemann, W. & Haferlach, T. (2004) FLT3 length mutations as marker for follow-up studies in acute myeloid leukaemia. *Acta Haematol*, **112**, 68-78.
- Scholl, S., Krause, C., Loncarevic, I.F., Muller, R., Kunert, C., Wedding, U., Sayer, H.G., Clement, J.H. & Hoffken, K. (2005) Specific detection of Flt3 point mutations by highly sensitive real-time polymerase chain reaction in acute myeloid leukemia. *J Lab Clin Med*, **145**, 295-304.
- Schwable, J., Choudhary, C., Thiede, C., Tickenbrock, L., Sargin, B., Steur, C., Rehage, M., Rudat, A., Brandts, C., Berdel, W.E., Muller-Tidow, C. & Serve, H. (2005) RGS2 is an important target gene of Flt3-ITD mutations in AML and functions in myeloid differentiation and leukemic transformation. *Blood*, **105**, 2107-2114.
- Sheikhha, M.H., Awan, A., Tobal, K. & Liu Yin, J.A. (2003) Prognostic significance of FLT3 ITD and D835 mutations in AML patients. *Hematol J*, **4**, 41-46.
- Shih, L.Y., Huang, C.F., Wu, J.H., Lin, T.L., Dunn, P., Wang, P.N., Kuo, M.C., Lai, C.L. & Hsu, H.C. (2002) Internal tandem duplication of FLT3 in relapsed acute myeloid leukemia: a comparative analysis of bone marrow samples from 108 adult patients at diagnosis and relapse. *Blood*, **100**, 2387-2392.
- Shih, L.Y., Huang, C.F., Wu, J.H., Wang, P.N., Lin, T.L., Dunn, P., Chou, M.C., Kuo, M.C. & Tang, C.C. (2004) Heterogeneous patterns of FLT3 Asp(835) mutations in relapsed de novo acute myeloid leukemia: a comparative analysis of 120 paired diagnostic and relapse bone marrow samples. *Clin Cancer Res*, **10**, 1326-1332.
- Shih, L.Y., Kuo, M.C., Liang, D.C., Huang, C.F., Lin, T.L., Wu, J.H., Wang, P.N., Dunn, P. & Lai, C.L. (2003) Internal tandem duplication and Asp835 mutations

- of the FMS-like tyrosine kinase 3 (FLT3) gene in acute promyelocytic leukemia. *Cancer*, **98**, 1206-1216.
- Simon, M., Grandage, V.L., Linch, D.C. & Khwaja, A. (2005) Constitutive activation of the Wnt/beta-catenin signalling pathway in acute myeloid leukaemia. *Oncogene*, **24**, 2410-2420.
- Smith, B.D., Levis, M., Beran, M., Giles, F., Kantarjian, H., Berg, K., Murphy, K.M., Dausess, T., Allebach, J. & Small, D. (2004) Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. *Blood*, **103**, 3669-3676.
- Smith, M.L., Arch, R., Smith, L.L., Bainton, N., Neat, M., Taylor, C., Bonnet, D., Cavenagh, J.D., Andrew Lister, T. & Fitzgibbon, J. (2005) Development of a human acute myeloid leukaemia screening panel and consequent identification of novel gene mutation in FLT3 and CCND3. *Br J Haematol*, **128**, 318-323.
- Somervaille, T.C. & Cleary, M.L. (2006) Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell*, **10**, 257-268.
- Song, W.J., Sullivan, M.G., Legare, R.D., Hutchings, S., Tan, X., Kufrin, D., Ratajczak, J., Resende, I.C., Haworth, C., Hock, R., Loh, M., Felix, C., Roy, D.C., Busque, L., Kurnit, D., Willman, C., Gewirtz, A.M., Speck, N.A., Bushweller, J.H., Li, F.P., Gardiner, K., Poncz, M., Maris, J.M. & Gilliland, D.G. (1999) Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. *Nat Genet*, **23**, 166-175.
- Songyang, Z., Carraway, K.L., 3rd, Eck, M.J., Harrison, S.C., Feldman, R.A., Mohammadi, M., Schlessinger, J., Hubbard, S.R., Smith, D.P., Eng, C. & et al. (1995) Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. *Nature*, **373**, 536-539.
- Sotlar, K., Marafioti, T., Griesser, H., Theil, J., Aepinus, C., Jaussi, R., Stein, H., Valent, P. & Horny, H.P. (2000) Detection of c-kit mutation Asp 816 to Val in microdissected bone marrow infiltrates in a case of systemic mastocytosis associated with chronic myelomonocytic leukaemia. *Mol Pathol*, **53**, 188-193.
- Sperr, W.R., Walchshofer, S., Horny, H.P., Fodinger, M., Simonitsch, I., Fritzsche-Polanz, R., Schwarzing, I., Tschachler, E., Sillaber, C., Hagen, W., Geissler, K., Chott, A., Lechner, K. & Valent, P. (1998) Systemic mastocytosis associated with acute myeloid leukaemia: report of two cases and detection of the c-kit mutation Asp-816 to Val. *Br J Haematol*, **103**, 740-749.
- Spiekermann, K., Bagrintseva, K., Schoch, C., Haferlach, T., Hiddemann, W. & Schnittger, S. (2002) A new and recurrent activating length mutation in exon 20 of the FLT3 gene in acute myeloid leukemia. *Blood*, **100**, 3423-3425.
- Spiekermann, K., Bagrintseva, K., Schwab, R., Schmieja, K. & Hiddemann, W. (2003) Overexpression and constitutive activation of FLT3 induces STAT5 activation in primary acute myeloid leukemia blast cells. *Clin Cancer Res*, **9**, 2140-2150.
- Sternberg, D.W. & Licht, J.D. (2005) Therapeutic intervention in leukemias that express the activated fms-like tyrosine kinase 3 (FLT3): opportunities and challenges. *Curr Opin Hematol*, **12**, 7-13.
- Stirewalt, D.L., Kopecky, K.J., Meshinchi, S., Appelbaum, F.R., Slovak, M.L., Willman, C.L. & Radich, J.P. (2001) FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. *Blood*, **97**, 3589-3595.
- Stirewalt, D.L., Meshinchi, S., Kussick, S.J., Sheets, K.M., Pogosova-Agadjanyan, E., Willman, C.L. & Radich, J.P. (2004) Novel FLT3 point mutations within exon 14 found in patients with acute myeloid leukaemia. *Br J Haematol*, **124**, 481-484.

- Stirewalt, D.L. & Radich, J.P. (2003) The role of FLT3 in haematopoietic malignancies. *Nat Rev Cancer*, **3**, 650-665.
- Stone, R., Fischer, T., Paquette, R., Schiller, G., Schiffer, C., Ehninger, G., Cortes, J., Kantarjian, H., DeAngelo, D., Massimini, G., Li, X., Phillips, P. & Giles, F. (2006) Phase IB Study of PKC412, an Oral FLT3 Kinase Inhibitor, in Sequential and Simultaneous Combinations with Daunorubicin and Cytarabine (DA) Induction and High-Dose Cytarabine Consolidation in Newly Diagnosed Adult Patients (pts) with Acute Myeloid Leukemia (AML) under Age 61. *Blood*, **108**, Abstract 157.
- Stone, R., Fischer, T., Paquette, R., Schiller, G., Schiffer, C., Ehninger, G., Cortes, J., Kantarjian, H., DeAngelo, D., Yu, R., Zhang, L., Cohen, P., Wang, Y., Phillips, P. & Giles, F. (2005a) Phase IB Study of PKC412, an Oral FLT3 Kinase Inhibitor, in Sequential and Simultaneous Combinations with Daunorubicin and Cytarabine (DA) Induction and High-Dose Cytarabine Consolidation in Newly Diagnosed Patients with AML. *Blood*, **106**, Abstract 404.
- Stone, R.M. (2007) Novel therapeutic agents in acute myeloid leukemia. *Exp Hematol*, **35**, 163-166.
- Stone, R.M., DeAngelo, D.J., Klimek, V., Galinsky, I., Estey, E., Nimer, S.D., Grandin, W., Lebwohl, D., Wang, Y., Cohen, P., Fox, E.A., Neuberg, D., Clark, J., Gilliland, D.G. & Griffin, J.D. (2005b) Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. *Blood*, **105**, 54-60.
- Summers, K., Stevens, J., Kakkas, I., Smith, M., Smith, L.L., Macdougall, F., Cavenagh, J., Bonnet, D., Young, B.D., Lister, T.A. & Fitzgibbon, J. (2007) Wilms' tumour 1 mutations are associated with FLT3-ITD and failure of standard induction chemotherapy in patients with normal karyotype AML. *Leukemia*, **21**, 550-551; author reply 552.
- Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E.S., Pileri, S.A., Stein, H., Theile, J. & Vardiman, J.W. (2008) WHO Classification of Tumours. of Haematopoietic and Lymphoid Tissues. *IARC Press*, 109-138.
- Tamburini, J., Elie, C., Bardet, V., Chapuis, N., Park, S., Broet, P., Cornillet-Lefebvre, P., Lioure, B., Ugo, V., Blanchet, O., Ifrah, N., Witz, F., Dreyfus, F., Mayeux, P., Lacombe, C. & Bouscary, D. (2007) Constitutive phosphoinositide 3-kinase/Akt activation represents a favorable prognostic factor in de novo acute myelogenous leukemia patients. *Blood*, **110**, 1025-1028.
- Tauro, S., Craddock, C., Peggs, K., Begum, G., Mahendra, P., Cook, G., Marsh, J., Milligan, D., Goldstone, A., Hunter, A., Khwaja, A., Chopra, R., Littlewood, T., Peniket, A., Parker, A., Jackson, G., Hale, G., Cook, M., Russell, N. & Mackinnon, S. (2005) Allogeneic stem-cell transplantation using a reduced-intensity conditioning regimen has the capacity to produce durable remissions and long-term disease-free survival in patients with high-risk acute myeloid leukemia and myelodysplasia. *J Clin Oncol*, **23**, 9387-9393.
- Taussig, D.C., Davies, A.J., Cavenagh, J.D., Oakervee, H., Syndercombe-Court, D., Kelsey, S., Amess, J.A., Rohatiner, A.Z., Lister, T.A. & Barnett, M.J. (2003) Durable remissions of myelodysplastic syndrome and acute myeloid leukemia after reduced-intensity allografting. *J Clin Oncol*, **21**, 3060-3065.
- Teller, S., Kramer, D., Bohmer, S.A., Tse, K.F., Small, D., Mahboobi, S., Wallrapp, C., Beckers, T., Kratz-Albers, K., Schwable, J., Serve, H. & Bohmer, F.D. (2002) Bis(1H-2-indolyl)-1-methanones as inhibitors of the hematopoietic tyrosine kinase Flt3. *Leukemia*, **16**, 1528-1534.
- Thiede, C., Bloomfield, C.D., Lo Coco, F., Frohling, S., Hiddemann, W., Krauter, J., Schaich, M., Whitman, S.P., Saglio, G., Dohner, K., Spiekermann, K.,

- Schnittger, S., Ganser, A., Heil, G., Buchner, T., Dohner, H. & Larson, R.A. (2007) The high prevalence of FLT3-ITD mutations is associated with the poor outcome in adult patients with t(6;9)(p23;q34) positive AML - results of an international metaanalysis. *Blood*, **110**, a761.
- Thiede, C., Steudel, C., Mohr, B., Schaich, M., Schakel, U., Platzbecker, U., Wermke, M., Bornhauser, M., Ritter, M., Neubauer, A., Ehninger, G. & Illmer, T. (2002) Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood*, **99**, 4326-4335.
- Tickenbrock, L., Schwable, J., Wiedehege, M., Steffen, B., Sargin, B., Choudhary, C., Brandts, C., Berdel, W.E., Muller-Tidow, C. & Serve, H. (2005) Flt3 tandem duplication mutations cooperate with Wnt signaling in leukemic signal transduction. *Blood*, **105**, 3699-3706.
- Trudel, S., Li, Z.H., Wei, E., Wiesmann, M., Chang, H., Chen, C., Reece, D., Heise, C. & Stewart, A.K. (2005) CHIR-258, a novel, multitargeted tyrosine kinase inhibitor for the potential treatment of t(4;14) multiple myeloma. *Blood*, **105**, 2941-2948.
- Tse, K.F., Novelli, E., Civin, C.I., Bohmer, F.D. & Small, D. (2001) Inhibition of FLT3-mediated transformation by use of a tyrosine kinase inhibitor. *Leukemia*, **15**, 1001-1010.
- Veiby, O.P., Lyman, S.D. & Jacobsen, S.E. (1996) Combined signaling through interleukin-7 receptors and flt3 but not c-kit potently and selectively promotes B-cell commitment and differentiation from uncommitted murine bone marrow progenitor cells. *Blood*, **88**, 1256-1265.
- Virappane, P., Gale, R., Hills, R., Kakkas, I., Summers, K., Stevens, J., Allen, C., Green, C., Quentmeier, H., Drexler, H., Burnett, A., Linch, D., Bonnet, D., Lister, T.A. & Fitzgibbon, J. (2008) Mutation of the Wilms' Tumor 1 Gene Is a Poor Prognostic Factor Associated With Chemotherapy Resistance in Normal Karyotype Acute Myeloid Leukemia: The United Kingdom Medical Research Council Adult Leukaemia Working Party. *J Clin Oncol*.
- Wang, L., Lin, D., Zhang, X., Chen, S., Wang, M. & Wang, J. (2005) Analysis of FLT3 internal tandem duplication and D835 mutations in Chinese acute leukemia patients. *Leuk Res*, **29**, 1393-1398.
- Weinstein, I.B. & Joe, A. (2008) Oncogene addiction. *Cancer Res*, **68**, 3077-3080; discussion 3080.
- Weisberg, E., Boulton, C., Kelly, L.M., Manley, P., Fabbro, D., Meyer, T., Gilliland, D.G. & Griffin, J.D. (2002) Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. *Cancer Cell*, **1**, 433-443.
- Weisberg, E., Roesel, J., Bold, G., Furet, P., Jiang, J., Cools, J., Wright, R.D., Nelson, E., Barrett, R., Ray, A., Moreno, D., Hall-Meyers, E., Stone, R., Galinsky, I., Fox, E., Gilliland, G., Daley, J.F., Lazo-Kallanian, S., Kung, A.L. & Griffin, J.D. (2008) Antileukemic effects of the novel, mutant FLT3 inhibitor NVP-AST487: effects on PKC412-sensitive and -resistant FLT3-expressing cells. *Blood*, **112**, 5161-5170.
- Wheatley, K., Burnett, A.K., Goldstone, A.H., Gray, R.G., Hann, I.M., Harrison, C.J., Rees, J.K., Stevens, R.F. & Walker, H. (1999) A simple, robust, validated and highly predictive index for the determination of risk-directed therapy in acute myeloid leukaemia derived from the MRC AML 10 trial. United Kingdom Medical Research Council's Adult and Childhood Leukaemia Working Parties. *Br J Haematol*, **107**, 69-79.

- Whitman, S.P., Archer, K.J., Feng, L., Baldus, C., Becknell, B., Carlson, B.D., Carroll, A.J., Mrozek, K., Vardiman, J.W., George, S.L., Kolitz, J.E., Larson, R.A., Bloomfield, C.D. & Caligiuri, M.A. (2001) Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: a cancer and leukemia group B study. *Cancer Res*, **61**, 7233-7239.
- Whitman, S.P., Ruppert, A.S., Radmacher, M.D., Mrozek, K., Paschka, P., Langer, C., Baldus, C.D., Wen, J., Racke, F., Powell, B.L., Kolitz, J.E., Larson, R.A., Caligiuri, M.A., Marcucci, G. & Bloomfield, C.D. (2008) FLT3 D835/I836 mutations are associated with poor disease-free survival and a distinct gene-expression signature among younger adults with de novo cytogenetically normal acute myeloid leukemia lacking FLT3 internal tandem duplications. *Blood*, **111**, 1552-1559.
- Wiemels, J.L., Cazzaniga, G., Daniotti, M., Eden, O.B., Addison, G.M., Masera, G., Saha, V., Biondi, A. & Greaves, M.F. (1999) Prenatal origin of acute lymphoblastic leukaemia in children. *Lancet*, **354**, 1499-1503.
- Wierenga, A.T., Schepers, H., Moore, M.A., Vellenga, E. & Schuringa, J.J. (2006) STAT5-induced self-renewal and impaired myelopoiesis of human hematopoietic stem/progenitor cells involves down-modulation of C/EBPalpha. *Blood*, **107**, 4326-4333.
- Xiao, W. & Oefner, P.J. (2001) Denaturing high-performance liquid chromatography: A review. *Hum Mutat*, **17**, 439-474.
- Xu, F., Taki, T., Yang, H.W., Hanada, R., Hongo, T., Ohnishi, H., Kobayashi, M., Bessho, F., Yanagisawa, M. & Hayashi, Y. (1999) Tandem duplication of the FLT3 gene is found in acute lymphoblastic leukaemia as well as acute myeloid leukaemia but not in myelodysplastic syndrome or juvenile chronic myelogenous leukaemia in children. *Br J Haematol*, **105**, 155-162.
- Yamamoto, Y., Kiyoi, H., Nakano, Y., Suzuki, R., Kodera, Y., Miyawaki, S., Asou, N., Kuriyama, K., Yagasaki, F., Shimazaki, C., Akiyama, H., Saito, K., Nishimura, M., Motoji, T., Shinagawa, K., Takeshita, A., Saito, H., Ueda, R., Ohno, R. & Naoe, T. (2001) Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood*, **97**, 2434-2439.
- Yanada, M., Matsuo, K., Suzuki, T., Kiyoi, H. & Naoe, T. (2005) Prognostic significance of FLT3 internal tandem duplication and tyrosine kinase domain mutations for acute myeloid leukemia: a meta-analysis. *Leukemia*, **19**, 1345-1349.
- Yang, X., Liu, L., Sternberg, D., Tang, L., Galinsky, I., DeAngelo, D. & Stone, R. (2005) The FLT3 Internal tandem duplication mutation prevents apoptosis in interleukin-3-deprived BaF3 cells due to protein kinase A and ribosomal S6 kinase 1-mediated BAD phosphorylation at serine 112. *Cancer Res*, **65**, 7338-7347.
- Yee, K.W., O'Farrell, A.M., Smolich, B.D., Cherrington, J.M., McMahon, G., Wait, C.L., McGreevey, L.S., Griffith, D.J. & Heinrich, M.C. (2002) SU5416 and SU5614 inhibit kinase activity of wild-type and mutant FLT3 receptor tyrosine kinase. *Blood*, **100**, 2941-2949.
- Yokota, S., Kiyoi, H., Nakao, M., Iwai, T., Misawa, S., Okuda, T., Sonoda, Y., Abe, T., Kahsima, K., Matsuo, Y. & Naoe, T. (1997) Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. *Leukemia*, **11**, 1605-1609.
- Zhang, S. & Broxmeyer, H.E. (1999) p85 subunit of PI3 kinase does not bind to human Flt3 receptor, but associates with SHP2, SHIP, and a tyrosine-phosphorylated

- 100-kDa protein in Flt3 ligand-stimulated hematopoietic cells. *Biochem Biophys Res Commun*, **254**, 440-445.
- Zhang, S. & Broxmeyer, H.E. (2000) Flt3 ligand induces tyrosine phosphorylation of gab1 and gab2 and their association with shp-2, grb2, and PI3 kinase. *Biochem Biophys Res Commun*, **277**, 195-199.
- Zhang, S., Fukuda, S., Lee, Y., Hangoc, G., Cooper, S., Spolski, R., Leonard, W.J. & Broxmeyer, H.E. (2000) Essential role of signal transducer and activator of transcription (Stat)5a but not Stat5b for Flt3-dependent signaling. *J Exp Med*, **192**, 719-728.
- Zhang, S., Mantel, C. & Broxmeyer, H.E. (1999) Flt3 signaling involves tyrosyl-phosphorylation of SHP-2 and SHIP and their association with Grb2 and Shc in Baf3/Flt3 cells. *J Leukoc Biol*, **65**, 372-380.
- Zhang, W., Konopleva, M., Shi, Y.X., McQueen, T., Harris, D., Ling, X., Estrov, Z., Quintas-Cardama, A., Small, D., Cortes, J. & Andreeff, M. (2008) Mutant FLT3: a direct target of sorafenib in acute myelogenous leukemia. *J Natl Cancer Inst*, **100**, 184-198.
- Zhao, M., Kiyoi, H., Yamamoto, Y., Ito, M., Towatari, M., Omura, S., Kitamura, T., Ueda, R., Saito, H. & Naoe, T. (2000) In vivo treatment of mutant FLT3-transformed murine leukemia with a tyrosine kinase inhibitor. *Leukemia*, **14**, 374-378.
- Zheng, R., Friedman, A.D., Levis, M., Li, L., Weir, E.G. & Small, D. (2004a) Internal tandem duplication mutation of FLT3 blocks myeloid differentiation through suppression of C/EBPalpha expression. *Blood*, **103**, 1883-1890.
- Zheng, R., Friedman, A.D. & Small, D. (2002) Targeted inhibition of FLT3 overcomes the block to myeloid differentiation in 32Dcl3 cells caused by expression of FLT3/ITD mutations. *Blood*, **100**, 4154-4161.
- Zheng, R., Levis, M., Piloto, O., Brown, P., Baldwin, B.R., Gorin, N.C., Beran, M., Zhu, Z., Ludwig, D., Hicklin, D., Witte, L., Li, Y. & Small, D. (2004b) FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells. *Blood*, **103**, 267-274.
- Zhou, J., Khng, J., Jasinghe, V.J., Bi, C., Neo, C.H., Pan, M., Poon, L.F., Xie, Z., Yu, H., Yeoh, A.E., Lu, Y., Glaser, K.B., Albert, D.H., Davidsen, S.K. & Chen, C.S. (2008) In vivo activity of ABT-869, a multi-target kinase inhibitor, against acute myeloid leukemia with wild-type FLT3 receptor. *Leuk Res*, **32**, 1091-1100.